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97. A STUDY OF PROGRESS OF LACTATION IN RELATION TO THE MILK YIELD AND THE BUTTERFAT PERCENTAGE OF MILK PRODUCED BY COWS OF SHORTHORN TYPE

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(With 2 Figures.)

INTRODUCTION.

THE mean yield of milk and the mean butterfat percentage of milk given by individual cows at successive afternoon and morning visits of the Milk Recorder have been determined. From the figures so obtained, the yield of butterfat and the correlation coefficient of milk yield and butterfat percentage have been calculated.

It is generally accepted that the milk yield tends to rise to a maximum within the first eight weeks after calving and then to decline, more or less slowly, subject to minor fluctuations due to variations in health, weather conditions, feeding and general management. On the other hand, the butterfat percentage tends to fall to a minimum some weeks after calving and then to rise, more or less steadily, to a maximum at the end of the lactation. An attempt has been made to determine whether or not such general trends can be traced in the data under review.

DATA.

The data consist of the recorded yield of milk, in pounds, of some two thousand five hundred afternoon and morning weighings, together with the corresponding butterfat percentages. After the elimination of all lactations spoilt by lack of data on one or more occasions (*e.g.* due to such causes as illness or spillage of milk), the figures relating to the first five consecutive afternoon and morning tests of some five hundred lactations remain to be examined.

The weights of milk were recorded and the samples for analysis were drawn by the official Milk Recorders at intervals of approximately six weeks over a period of five years.

The butterfat determinations were made by the Gerber method in the Milk Advisory Laboratory of the Cambridge School of Agriculture.

Treatment of data.

The data were tabulated to show the milk yield and the corresponding butterfat percentage of all cows at each successive afternoon and morning visit of the Milk Recorder.

It was found that, while the number of records relating to each of the first four visits was approximately constant, the number at the fifth visit showed a drop of 11 per cent. after which the decline became more rapid. Although records of some cows continued to appear until the eighth or ninth visit, it was considered necessary, in view of the small number so continuing, to include only the first five tests.

A. *Milk yield.*

The mean yields at the afternoon and morning milking for each of the five visits are set out in Table I.

Table I. *Mean yield of milk in pounds.*

	Afternoon			Morning		
Test No.	No. of records	Mean yield	S.E. of mean	No. of records	Mean yield	S.E. of mean
1	523	15·44	0·22	522	22·34	0·28
2	523	13·89	0·20	522	20·22	0·25
3	523	11·64	0·18	522	17·54	0·24
4	523	9·81	0·15	522	15·31	0·21
5	454	8·45	0·15	456	13·41	0·22

It will be seen that the yield falls significantly with each successive test both for afternoon and morning milkings. The fact that no peak is shown is probably due to the somewhat irregular intervals between successive visits of the recorder; the average interval being six weeks with a range of from four to nine weeks. The interval between calving and the taking of the first test may vary within the limits of four to sixty-three days.

It may be remarked that the maximum mean daily yield at the first test is almost four gallons, while at the fifth test it is still above two gallons. Calculation of the mean after further intervals shows that it declines very slowly. This is probably due to the fact that it is chiefly cows of long staying power that appear in the records at this stage.

B. *Butterfat percentage.*

The butterfat percentages corresponding to the above means of yield are shown in Table II.

Table II. *Mean butterfat percentages.*

	Afternoon			Morning		
Test No.	No. of records	Mean %	S.E. of mean	No. of records	Mean %	S.E. of mean
1	523	4·21	0·04	522	3·16	0·03
2	523	4·01	0·03	522	3·07	0·02
3	523	4·02	0·03	522	3·16	0·02
4	523	4·09	0·03	522	3·25	0·02
5	454	4·16	0·04	456	3·37	0·03

It will be seen that the butterfat percentage falls from the first to the second test and subsequently rises to the fifth, in both afternoon and morning groups. In the afternoon the maximum mean butterfat percentage occurs at the first test, but in the morning the third test is approximately equal to the first, the fourth and fifth being higher.

An analysis of variance shows that the variance between the five tests in both afternoon and morning groups is significantly greater than that within tests ($P<0{\cdot}01$). Thus the difference between means is greater than could have occurred by chance; the general trend may therefore be regarded as significant even if the individual differences are not in all instances significant.

C. *Correlation of milk yield and butterfat percentage.*

The intervals selected for the table of frequency distribution were respectively 1 lb. for yield and 0·2 per cent. for butterfat. Thus, in the former, fractions one-quarter, one-half and three-quarters were credited to the next lower unit group giving an interval mean of $y=0{\cdot}375$ lb. With butterfat percentages an odd number in the first decimal place was grouped with the next lower even number, thus giving an interval mean of $x=0{\cdot}05$.

The coefficient of correlation for each test is given in Table III, together with its standard error and an estimate of its significance.

Table III. *Coefficient of correlation of milk yield and butterfat percentage.*

	Afternoon			Morning		
Test No.	r	S.E. of r	$\frac{r}{\text{S.E. of } r}$	r	S.E. of r	$\frac{r}{\text{S.E. of } r}$
1	−0·102	0·043	2·34	−0·088	0·043	2·02
2	−0·204	0·034	3·82	−0·109	0·043	2·56
3	−0·100	0·034	2·96	−0·210	0·042	5·02
4	−0·123	0·043	2·84	−0·182	0·042	4·30
5	−0·251	0·044	5·70	−0·184	0·045	4·00

It will be seen that there is a small negative correlation in every instance. Reference to an unpublished table[1] of values of N up to 500 (based on Table 5A of Fisher) shows that the coefficient of correlation is in all instances significant; the results of tests P.M. No. 1 and A.M. Nos. 1 and 2 giving values of $P<0{\cdot}05$ while for the remaining tests $P<0{\cdot}01$. The group figures for afternoon and morning respectively are as follows:

$$\text{Afternoon: } r=-0{\cdot}154\pm0{\cdot}019,$$
$$\text{Morning: } r=-0{\cdot}151\pm0{\cdot}019.$$

A graph showing regression of butterfat percentage about milk yield is given in Fig. 1.

D. *Yield of butterfat in pounds.*

An attempt has been made to calculate the mean yield of butterfat in pounds for afternoon and morning milking at each test. For this purpose the milk yield in pounds has been multiplied by the figure for butterfat and the

[1] Private communication from Dr Wishart.

product divided by 100. A correction has been applied to compensate for the correlation already found. The formula is as follows:

$$\text{Yield of fat in pounds} = \frac{(\text{fat reading} \times \text{milk yield in pounds})}{100} + r\frac{(\text{S.D. of fat} \times \text{S.D. of yield})}{100}.$$

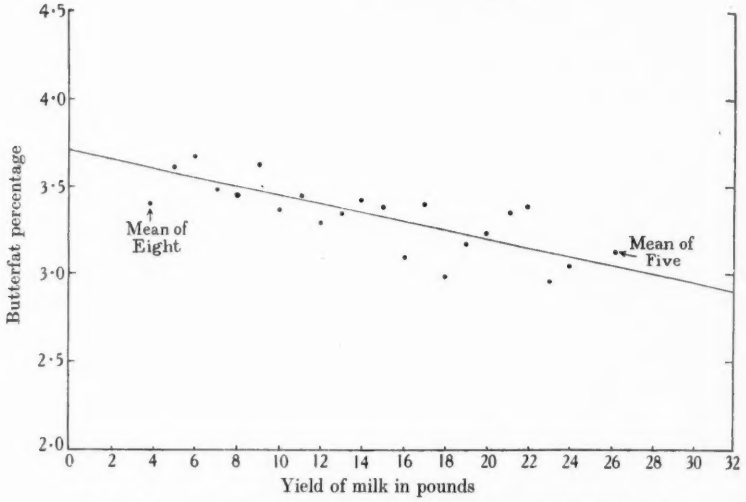

Fig. 1. Regression of butterfat percentage about milk yield. Fifth test. Morning samples. $r = -0{\cdot}184 \pm 0{\cdot}045$.

The calculated yield of butterfat in pounds, together with the ratio of production at the afternoon and morning milkings, are set out in Table IV.

Table IV. *Yield of butterfat in pounds.*

Test No.	lb. fat P.M.	lb. fat A.M.	Ratio A.M. : P.M.
1	0·645	0·703	1·089 : 1
2	0·552	0·617	1·118 : 1
3	0·470	0·549	1·167 : 1
4	0·398	0·492	1·235 : 1
5	0·327	0·446	1·364 : 1

It will be seen that, although the mean butterfat percentage of the morning milk is always lower than that of the afternoon milk, the total yield of butterfat is in every instance higher in the morning than in the afternoon, due to the greater milk yield at the morning milking. It will be noted, further, that the divergence between the yield of butterfat at the morning and evening milking becomes more marked as lactation progresses, within the limits set by the data under examination.

The general trend of milk yield, butterfat percentage and yield of butterfat are more clearly seen in Fig. 2, where they are plotted against time intervals between tests.

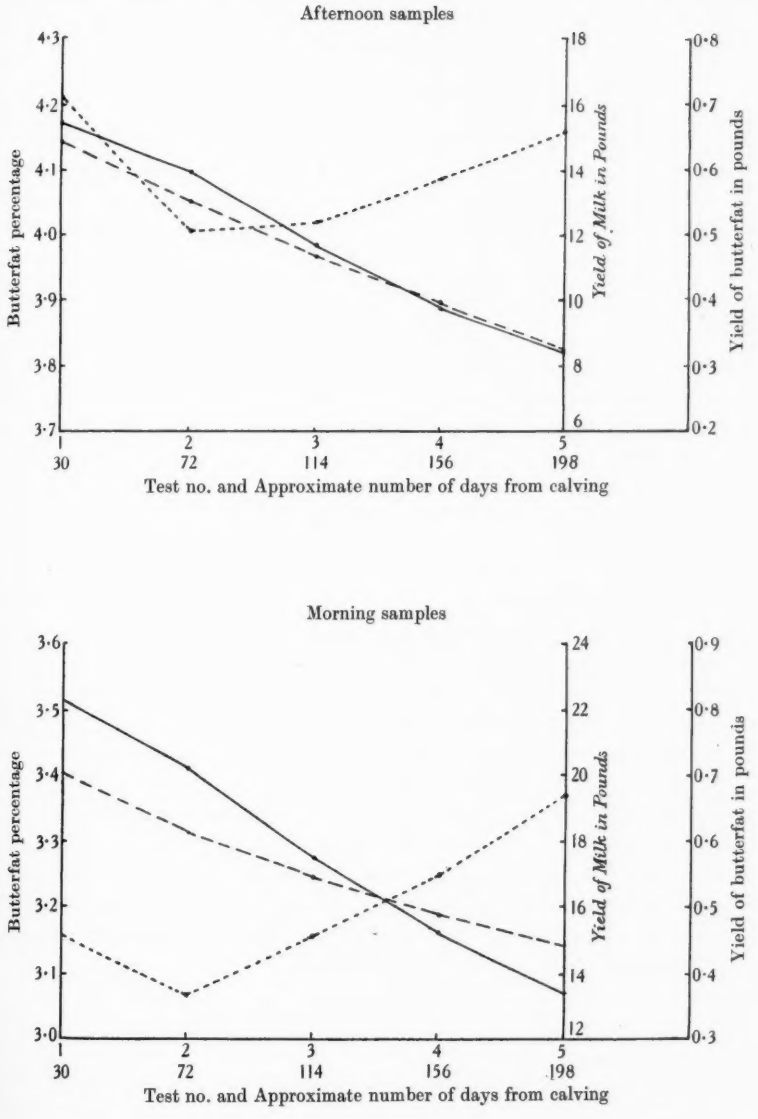

Fig. 2. Effect of progress of lactation on milk yield, butterfat percentage and yield of butterfat.

E. *Relative values at afternoon and morning milking.*

The ratios of milk yield, butterfat percentage and yield of butterfat respectively at the afternoon and morning milkings are shown in Table V.

Table V. *Ratio afternoon to morning.*

Test No.	Milk yield	Butterfat (%)	Butterfat yield
1	1 : 1·446	1 : 0·752	1 : 1·089
2	1 : 1·456	1 : 0·766	1 : 1·118
3	1 : 1·503	1 : 0·788	1 : 1·167
4	1 : 1·560	1 : 0·794	1 : 1·235
5	1 : 1·586	1 : 0·810	1 : 1·364

It will be seen from Table V that there is a definite and progressive change in relationship of milk yield, fat percentage and yield of fat as between afternoon and morning milk. In all three columns the ratio of afternoon to morning falls progressively from the first test to the fifth. While not necessarily significant, the regular nature of the change is, at least, suggestive and is based on figures which have been shown to be themselves significant.

Discussion.

(1) *Reliability of data.*

The data are relatively unselected—the only requirement being that of results from officially recorded cows of Shorthorn type milked twice daily and having at least five successive tests. Results obtained from nine separate herds were included. Further, the first test visit occurred with a fairly constant frequency in each month of the year, with the exception of August. As to the practice of accepting the results obtained for butterfat percentage at a single test as representative of that during the whole period of six weeks, it has been shown at the National Institute for Research in Dairying (1) that the error introduced did not exceed 0·2 per cent. fat. Houston and Hale (2) found a maximum error of calculated butterfat percentage at six-weekly intervals equivalent to 12·41 per cent. of the true butterfat percentage. They point out, however, that in most instances errors of opposite sign cancel out and thus considerably reduce the final error.

(2) *Period of maximum milk yield.*

The long and somewhat irregular intervals between tests preclude any attempt being made to fix accurately the time of peak yield. This undoubtedly varies with management and with breed. Thus Sanders (3) shows a peak yield occurring between the third and the sixth week. Bartlett (4), working with Shorthorn cows under conditions of good management, places the peak about the fiftieth day. McCandlish (5) gives the first month as the period of maximum yield for Guernsey and Jersey cows, but for Ayrshires and Holsteins he finds it occurring during the second month. If, in the data under review, it is

assumed that approximately half the cows are first tested before and half after the mid-point of the interval, viz. approximately thirty days from calving, then the peak may in fact have occurred about the time of the first test.

(3) *Period of minimum butterfat percentage.*

Bartlett (4) shows that the minimum butterfat percentage occurs about the eightieth day for afternoon milk and about the fiftieth day for morning milk.

The shape of the afternoon curve (Fig. 2) is not altogether inconsistent with a minimum occurring actually later than the second test, *i.e.* later than the seventy-second day. The minimum for morning milk appears fairly sharp and occurs at about the second test. From the data under review, it is clear that there is a lag between the date of maximum milk production and that of minimum butterfat percentage. It is suggested that such a lag would naturally be more clearly defined in a larger and less selected population than in the single herd quoted by Bartlett. Thus McCandlish (5) gives figures which show that a lag occurs with cows of the Ayrshire, Holstein and Guernsey breeds, but the Jersey appears to give both maximum milk yield and minimum butterfat percentage during the first month of the lactation.

(4) *Correlation of milk yield and butterfat percentage.*

It would appear that most of the work on this subject has been based on a period of seven days or of one year. This applies to the work of Roberts (6) and of Gaines and Davidson (7). The latter give the coefficient of correlation in the case of the least selected populations, viz. Holstein and Jersey Cow Testing Associations, as $r = -0{\cdot}198 \pm 0{\cdot}012$ and $r = -0{\cdot}212 \pm 0{\cdot}021$ respectively. While the figures obtained in the present investigation are of somewhat the same order, they are not strictly comparable, since they are based on a number of isolated tests in each lactation and not on the total figures for the lactation.

It is possible, but by no means certain, that a somewhat higher coefficient of correlation might have been obtained if it had been possible to carry the tests beyond the two-hundredth day.

Summary.

1. The mean and the standard error of the mean of milk yield and of butterfat percentage have been determined for each of the first five successive Milk Recording Society tests of both afternoon and morning milk.

2. The yield of butterfat and the coefficient of correlation of milk yield and butterfat percentage at each test have been calculated.

3. The relationship of successive test results and of morning and evening results at the same period is discussed.

ACKNOWLEDGMENTS.

It is desired to record the thanks due to Dr H. G. Sanders for help with the statistical work and to Dr J. Wishart for criticisms and suggestions. Thanks are due also to the Milk Recording Society concerned for permission to make use of the data for publication.

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98. THE USE OF SPECIAL LITMUS MILK MEDIA FOR THE DIAGNOSTIC CULTURE OF LACTIC ACID BACTERIA: (1) DEXTROSE LITMUS MILK, (2) YEAST EXTRACT LITMUS MILK, AND (3) YEAST EXTRACT DEXTROSE LITMUS MILK

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Litmus milk is probably the most useful medium to the dairy bacteriologist, since it indicates fairly accurately what will be the action upon milk of the micro-organism under investigation. In spite of the advantages of synthetic indicators, such as brom-cresol-purple, litmus is still used because it gives a greater variety of reactions than a synthetic indicator. Thus the reducing power of a culture is adequately indicated by litmus, the normal E_h being about $-0{\cdot}09$ volt at *p*H 5 (1). Some lactic acid bacteria, *e.g. Str. lactis*, readily reduce litmus, while others do not or only very slightly, *e.g. Sbm. plantarum*. Plain litmus milk is, however, an inadequate medium for some bacteria, which either clot the medium only after several weeks growth, or never produce sufficient acid to bring about this change (2). It has been found by the author that such bacteria grow rapidly in litmus milk to which has been added dextrose, or yeast extract, or both. Some strains are particularly sensitive to dextrose (group 1) and some to the yeast extract (group 2), while others grow more rapidly when both are present than with either addition alone (group 3).

It would appear probable that in the first group are to be found lactic acid bacteria that have lost (or never possessed) the power of synthesising lactase, but can readily ferment dextrose, while in the second group the bacteria are unable to form their enzymes readily from the constituents of the milk but are able to synthesise lactase readily from the yeast substances. The bacteria of the third group apparently require the yeast extract to synthesise the dextrose-splitting enzyme and cannot form lactase even with these additional nutritive substances.

In Table I are given the times required by type cultures to clot the milk with various additions. The media were inoculated with equal parts of a uniform suspension of bacteria in 0·9 per cent. NaCl. The same separated milk was used for making all four media.

Since observations were not made every day, the times given in some cases represent a longer period than was actually required to clot the milk. However, the data are sufficient to show the marked differences in the rates of acid production in the various media. The *Thermobacteria* and the pathogenic streptococci were incubated at 37° C., the others at 32° C.

Table I.

	No. of days required to clot milk			
Parasitic streptococci:	L.M.	D.L.M.	Y.L.M.	Y.D.L.M.
S. faecium 2703 [Hucker]	8	6	1	1
S. lactis 662 [Thaysen]	>70*	4	2	2
S. lactis [Orla-Jensen]	1	1	1–	1
S. cremoris [Orla-Jensen]	4–9	4	2	2
S. liquefaciens 2705 [Hucker]	5	5	5	5
S. bovis 2701 [Hucker]	27	9	2	2
S. glycerinaceus	26	9	26	2
S. lactis (Caramel flavour) 3252 [Sadler]	2	2	2	2
S. faecalis 370 [Douglas]	11	9	2	2
Enterococcus 2707 [Tissier]	9	9	2	2
S. lactis 2700 [Hucker]	>72*	3	>72*	1
Enterococcus 775 [Tissier]	9	9	2	2
Pathogenic streptococci:				
S. haemolyticus [325 Avery]	>20	>20	2	2
S. viridans [1080 Cowan]	44	44	6	6
S. pyogenes [2400 Rettger]	>12	12	2	1
S. epidemicus [2297 Frost]	2	1	1	1
S. haemolyticus [McL.]	>19	>19	9	9
Betacocci:				
S. paracitrovorus D22 [Hammer]	>54	46	31	6
DN2 [Hammer]	>52	37	>37	6
Leuc. mesenteroides 3274 [Kluyver]	>46	9	>46	2
Bc. arabinosaceus 2706 [Hucker]	>52	52	6	2
Tetracocci:				
T. casei 2699 [Hucker]	4–9	4–9	4–9	4–9
T. liquefaciens 3251 [Sadler]	2–3	2–3	1	1
Thermobacteria:				
Tbm. cereale (*L. Delbrücki*) 4033	>60	6	>60	6
L. Leichmanii 4035	>60	13	>60	6
L. acidophilus 1724 [Rettger]	2–6	2–6	2–6	2–6
L. acidophilus 1899 [Kopeloff]	2–6	2–6	2–6	2–6
L. Delbrücki [Berlin]	2–6	2–6	2–6	2–6
L. acidophilus (Commercial)	2–6	2–6	2–6	2–6
L. acidophil-aerogenes 4034	>60	23	>60	6
Tbm. Bulgaricus [Orla-Jensen]	1	1	1	1
[Fildes]	>17*	>17*	5	5
[Ritter]	>19*	>19*	>19*	7
Tbm. lactis [Ritter]	2	2	1	1
L. odontolyticus 1407 [McIntosh]	19	27	5	5
L. bifidus 2797 [Cruickshank]	>19	>19	12	5
Tbm. Jughurt [Orla-Jensen]	2	2	2	2
L. odontolyticus 1406 [McIntosh]	12	19	5	2
L. bulgaricus (Commercial)	3	3	1	1
Tbm. helveticum 4113	3	3	3	3
L. bulgaricus 2889 [Metchnikoff]	5	5	5	5
Streptobacteria:				
Sbm. casei [Orla-Jensen]	7	12	5	5
,, ,, [Ritter]	5	5	5	5
3253 [Sadler]	5	6–16	5	5
Sbm. plantarum 4125	>67	>67	7	4
,, ,, 1–4 [Davis]	22	22	5	5
,, ,, 1–8 [Davis]	30	5	12	5
,, ,, 2–2 [Davis]	22	22	5	5

L.M. = Litmus milk. Y.L.M. = Yeast litmus milk.
D.L.M. = Dextrose litmus milk. Y.D.L.M. = Yeast dextrose litmus milk.

* Culture in attenuated condition.

Table I (*continued*).

	No. of days required to clot milk			
Streptobacteria (*continued*):	L.M.	D.L.M.	Y.L.M.	Y.D.L.M
Sbm. plantarum (intermediate) 2–9 [Davis]	12	12	5	5
Sbm. casei 2–11 [Davis]	5	5	5	5
2–13 [Davis]	5	5	5	5
Sbm. plantarum 3254 [Sadler]	5	5	5	5
Betabacteria:				
Bbm. caucasicum [Orla-Jensen]	45	45	10	10
L. fermentatae 4036	67	44	67	5
Bbm. breve R 3 [Ritter]	47	47	10	10
× R 4 [Ritter]	10	10	10	10
L. pentoaceticus 947	>67	67	12	5
„ „ 4037	30	7	22	5
Bbm. longum R 1 [Ritter]	45	>45	10	10
„ „ R 2 [Ritter]	>45	>45	10	10

In considering the data it is necessary to distinguish between differences due to the true (*i.e.* permanent) character of the organism, and those brought about by attenuation due to long culture on artificial media, inoculation from aged cultures and similar causes (3). The classification of Orla-Jensen (2) has been used throughout this paper. Type cultures received under other names are given as received. The three or four figure numbers after cultures are those of the National Collection of Type Cultures, Lister Institute, London.

From an examination of the table it is seen that the bacteria studied may be considered in four groups:

(1) Bacteria unaffected by the addition of dextrose or yeast extract:

(*a*) Typical milk bacteria, *e.g. Str. lactis*, the milk *Thermobacteria*, and *Sbm. casei*.

(*b*) Bacteria producing exogenous proteolytic enzymes, *e.g. Str. liquefaciens*, and the *Tetracocci* (the *Str. epidemicus* 2297 was atypical in that it peptonised milk and hence falls in this group).

(2) Bacteria stimulated by the presence of dextrose:

(*a*) Typical milk bacteria in an attenuated condition and producing lactase with difficulty, *e.g. Str. lactis* 2700.

(*b*) Plant strains, *e.g. Leuc. mesenteroides* 3274, *L. Delbrücki* 4033 and *L. pentoaceticus* 4037.

(3) Bacteria stimulated by the presence of yeast extract:

(*a*) Bacteria saprophytic in animals, *e.g. Str. faecium* (all strains), *Str. bovis*, *Leuc. odontolyticus* and *L. bifidus* 2797.

(*b*) Pathogenic streptococci, both α- and β-haemolytic types. (Orla-Jensen states that the pathogenic streptococci thrive very badly with yeast as a source of nitrogen (2). In this instance, however, the yeast may be regarded as a source of "growth factors" rather than as a source of nitrogen.)

(*c*) *Sbm. plantarum*.

(*d*) The *Betabacteria*.

(4) Bacteria stimulated more by the presence of both dextrose and yeast than by either alone:

(*a*) The *Betacocci.*

(*b*) Some *Betabacteria.*

It is evident from these data that valuable information as to the identity and previous habitat of any lactic acid bacterium may be obtained by culture in these four media.

In addition yeast dextrose litmus milk has been found to be the best medium for the general culture of the bacteria of importance in dairying. With the addition of chalk (about 1 g. in 10 ml. of medium) it makes an excellent medium for the propagation of stock cultures of the more fastidious bacteria. If chalk is not added, strains will die out faster in yeast dextrose litmus milk than in litmus milk on account of the greater vigour of growth and acid production.

Specification of media.

Litmus milk. Separated milk plus 1 per cent. purified litmus solution.

Dextrose litmus milk. Litmus milk plus 1 per cent. dextrose.

Yeast litmus milk. Litmus milk plus 5 per cent. autolysed yeast extract.

Yeast dextrose litmus milk. Litmus milk plus 1 per cent. dextrose plus 5 per cent. autolysed yeast extract.

Litmus solution. 120 g. litmus granules are extracted in 2000 ml. distilled water for ½ hour at 80° C. After filtering, glacial acetic acid is added until a bright red colour is obtained (15 ml.) and the liquid evaporated to a thick paste. This is transferred to a filter and washed with absolute alcohol. The residue is redissolved in 250 ml. distilled water at 70° C., filtered into a flask, and sterilised by steaming on 3 days for ½ hour.

Autolysed yeast extract. 2 kg. of pure pressed yeast are crumbled into a large wide-mouthed lidded glass jar, 0·1 per cent. sodium chloride added and stirred in well. The mass is then heated at 55° C. for 48 hours. It is necessary to stir the mass at intervals. The autolysed yeast is removed without shaking and filtered through a pulp filter. The filtrate is transferred to flasks in suitable quantities (*e.g.* 100 ml.) and autoclaved at 120° C. for 20 min.

I am indebted to Dr A. T. R. Mattick for permission to publish these formulae, and to Lt.-Col. E. C. G. Maddock and Dr W. Ritter and (*per* Dr Mattick) to Prof. P. Fildes and Prof. J. W. MacLeod for strains of lactic acid bacteria.

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99. THE EFFECT OF THE REACTION (*p*H) OF MILK ON THE DESTRUCTION OF MICRO-ORGANISMS BY HEAT

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In the extensive literature on the destruction of living micro-organisms by heat it is seldom that the *p*H of the medium is recorded. Much of the work has been carried out under widely different conditions of time and temperature and the age of the cells has varied, with the result that it is difficult, if not impossible, to compare one result with another.

Bigelow and Esty (1), however, have recognised the importance of reaction (*p*H) of the substrate and have shown that the greater the concentration of hydrogen ions, the more destructive is the effect upon spores, at the same temperature. Chick (2) found that additions of small quantities of acid or alkali to suspensions of *Bact. typhosum* in distilled water increased the rate of destruction under the influence of heat, but the effect of acid was much more marked than that of the alkali. It is generally supposed that when commercial milk is pasteurised at 62·8° C. for 30 min. the relative destruction of living organisms is as a rule greater in milk of very high bacterial content resulting from favourable temperature conditions than in milk containing comparatively few bacteria.

Thus it happens that pasteurisation efficiency as measured by the relative numbers of organisms surviving in rail-borne milk is greater in warm than in cold weather. The figures given in Table I tend to confirm this supposition. In view of the numerous inadequacies of the plate count it would be very unwise to place too great stress upon the differences between the figures in the two seasons, but the fact that all platings were carried out in quintuplicate on 1 per cent. milk agar and that the "efficiencies" are notably less in nearly all the winter cases, at least suggests a difference between the two series. There may be a number of reasons for the differences, and the age of the cells being heated and the conditions of growth are probably amongst them. The influence of various factors (including heat) tending to destroy cells has been shown by a number of workers to be greater in young cells and in old cells in the process of rejuvenescence than in others (3). In warm weather when growth of some types of organisms is rapid the proportion of young cells would be greater than in cold weather when growth is slow, and the effect of heat would be greater. In this connection the work of Dotterer (4) and Anderson and Meanwell (5) may be quoted. These authors found that milk produced under bad hygienic conditions contained more heat-resistant cells than milk produced under good conditions, and it is suggested that organisms derived from

dirty utensils, *i.e.* old cells or those grown below their optimum temperature were largely thermostable and survived pasteurisation.

Table I. *Machine-pasteurised milk.*

Date 1932	Colony count of raw milk per 1 ml.	Colony count of pasteurised milk per 1 ml.	Reduction in number of colonies %
July 10	55,000,000	1,500	99·99
,, 11	188,000,000	2,200	99·98
,, 17	163,000,000	14,500	99·99
,, 18	82,000,000	22,000	99·73
,, 25	128,000,000	6,400	99·99
,, 26	96,000,000	13,300	99·98
Aug. 1	272,000,000	7,300	99·99
,, 2	64,000	1,300	96·41
Dec. 1	18,500,000	24,000	99·87
,, 4	1,920,000	55,000	97·13
,, 5	234,000	11,000	95·09
,, 12	370,000	37,000	90·00
,, 14	260,000	7,400	97·26
,, 15	410,000	7,100	98·27
,, 18	720,000	4,000	99·45
,, 19	770,000	14,100	97·27

The predominating flora of milk of high bacterial count is usually acid-producing, and as numbers increase the natural *p*H of milk may be expected to decrease to a greater or lesser extent, depending on the buffer value of the milk.

It is, of course, not possible to pasteurise milk in which this process has gone too far. An idea of the range over which the *p*H may vary (*a*) in fresh mixed milk in which little or no bacterial growth has taken place, and (*b*) in commercial milk of very high bacterial count which survived the process of pasteurisation without apparent alteration, is given in Table II.

Table II.

Date	Approximate age of milk hours	Hydrogen-ion concentration	
		(*a*) Fresh milk*	(*b*) Commercial milk, high plate count†
1931–2	4	6·64–6·98	—
1932–3	4	6·57–7·00	—
1933–4 (summer period)	18–20	—	6·30–6·75

* Golding, Mackintosh and Mattick (*J. Dairy Res.* **6**, 6 (1935)). Range of a large number of determinations throughout the year.

† Authors' figures.

It seems that differences in *p*H of milk of different age, from different geological formations, from season to season and perhaps from breed to breed of cow may occur, and that these differences may be sufficient to contribute at least one of the reasons for the various "pasteurisation efficiencies" at 145° F. for 30 min., which are found in practice.

With these facts in mind the following experiments were carried out on samples of mixed milk from the districts which are shown in Table III.

Table III.

No. of Exp.	Place of origin (mixed milk)	No. of sub-samples	*p*H of milk just before pasteurisation	Colony count of raw milk before pasteurisation. Mean of duplicate determinations	Colony count of milk immediately after pasteurisation and cooling. Mean of duplicate determinations
I	Somersetshire	1	6·92	183,000	4,100
30. i. 34		*2	6·71	179,000	3,500
		3	6·64	210,000	3,600
		4	6·50	190,000	5,300
		5	6·36	156,000	2,300
II	Berkshire	1	6·81	1,240,000	2,250
31. i. 34		2	6·74	700,000	2,110
		*3	6·65	1,460,000	1,750
		4	6·48	820,000	2,000
		5	6·34	780,000	1,200
III	Somersetshire	1	6·85	880,000	67,100
1. ii. 34		*2	6·74	1,280,000	30,900
		3	6·52	780,000	9,300
		4	6·38	1,510,000	9,900
		5	6·24	1,120,000	8,800
IV	Somersetshire	1	6·92	3,120,000	14,400
6. ii. 34		*2	6·71	3,020,000	9,800
		3	6·60	4,340,000	9,400
		4	6·44	3,650,000	6,900
		5	6·30	3,610,000	9,100
V	Gloucestershire	1	6·87	113,000	11,660
7. ii. 34		2	6·76	86,000	5,020
		*3	6·64	82,000	2,180
		4	6·47	77,000	220
		5	6·27	90,000	200
VI	Somersetshire	1	6·97	2,520,000	33,000
8. ii. 34		2	6·78	2,800,000	20,800
		*3	6·60	2,900,000	14,300
		4	6·47	2,230,000	2,600
		5	6·25	2,600,000	2,100
VII	Somersetshire	1	6·95	6,560,000	127,200
13. ii. 34		*2	6·76	6,210,000	27,400
		3	6·62	5,620,000	6,400
		4	6·51	5,050,000	1,520
		5	6·31	5,420,000	1,250
VIII	Gloucestershire	1	7·00	30,500	1,770
14. ii. 34		2	6·85	28,500	1,030
		*3	6·66	22,900	520
		4	6·55	25,900	310
		5	6·41	27,700	510
IX	Somersetshire	1	6·72	9,600,000	6,910
15. ii. 34		*2	6·65	9,000,000	2,240
		3	6·59	8,700,000	1,330
		4	6·45	9,500,000	1,140
		5	6·37	10,800,000	730

* Indicates untouched raw milk.

Each sample of raw milk was well mixed and divided into five portions of 100 ml. Care was taken to treat all samples as uniformly as possible in every way.

One portion of each sample, indicated in Table III, was not treated in

any way, but to the other four subsamples small volumes of $N/10$ NaOH or $N/10$ HCl were added slowly and with constant shaking. The quantities to be added were roughly determined by reference to a titration curve of mixed milk constructed for the purpose. This curve showed the shift in pH of a representative sample of milk when any given quantity of acid or alkali was added. The titration curve could not, of course, be expected to give exact information for all samples of milk because of variations in buffer values, but it served as a useful guide. After addition of acid or alkali all samples were left for at least 2 hours at 18° C. to come to equilibrium, and the pH was then determined by means of the quinhydrone electrode. The figures given in the table are the results of these determinations, which are subject to a maximum error of $p\text{H} \pm 0{\cdot}03$ in the range over which the experiments extended.

Immediately after the pH determinations had been made the subsamples were pasteurised in stoppered test-tubes of resistance glass which had been in use for a considerable time, at 145° F. $\pm 0{\cdot}5°$ for 30 min. by immersion in a thermostat. The temperature in the tubes was controlled by observing a standard thermometer fixed in one of them. After pasteurisation the subsamples were cooled quickly and the dilutions immediately plated in duplicate on 1 per cent. milk agar.

Each subsample of raw milk was also plated in duplicate immediately prior to pasteurisation.

The results recorded in Table III show that in general the effect of heat, as reflected by numbers of surviving bacteria, is greatest when the pH is lowest, *i.e.* when the hydrogen-ion concentration is greatest. This appears to be so after the proper allowance has been made for sampling and other errors of the plate count. An inspection of the figures for the raw milk subsamples secured from any one sample gives an idea of the sampling error.

It is clear, however, that the data are not quite homogeneous. In Exps. I, II and IV, although the plate counts show the same general tendency to decrease with the pH, the differences are probably not significant. In the remaining experiments when the pH exceeds *c.* 6·5–6·6 the number of colonies on the plates is notably greater than at the lower pH values. The fact that the figures in Exps. I, II and IV do not exhibit the great differences found at the extremes of the pH range shown in the remaining experiments may be due to the type of flora or its vulnerability to heat, or to some other factor at present unknown.

It is not likely that the effect observed was due to Na or Cl ions, which were never added in quantities sufficient to disturb seriously the concentration of either in the ash of normal milk.

It may be counted as an advantage that the experiments were carried out on milk with its natural flora, so that they are not subject to the objections which may be raised against the use of cultures added artificially to milk and then heated.

DISCUSSION.

If the *p*H of milk normally varies over only a part of the range covered by the experiments it is possible that an amount of destruction of bacteria by heat at any given temperature in one milk may not justly be expected in another, simply because of differences in *p*H.

Further, in the preparation of such products as sterilised milk and cream or of products less drastically treated by heat it is not unlikely that differences in *p*H may explain irregularities which otherwise may appear to be anomalous. From time to time the presence of living tubercle bacilli in pasteurised milk is reported (6).

In view of the well-established fact that the official requirements for pasteurisation, viz. heating to 145–150° F. for 30 min. (7), allow a large margin of safety, these positive results are certainly due to defective plant or bad management (6). If, however, in practice it should happen that the required temperatures were not reached or maintained for the required time, or if during the holding period raw milk gained access to the tanks and was not held for the proper time, it is possible that part of the reason for the survival of tubercle bacilli on some occasions and not on others in the same plant, or in one plant and not in another of the same type, may lie in the relationship of the *p*H of the milk to the actual time and temperature of heating. This point is now under investigation.

SUMMARY.

It has been shown that alterations in the hydrogen-ion concentration of milk result in differences in the numbers of bacteria which survive the process of heating at 145° F. for 30 min. As the *p*H decreases the number of bacteria surviving also decreases. The possible bearings of these findings on various processes are discussed.

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100. THE PLATE COUNT OF MILK

EXPERIMENTAL ERRORS IN THE EXAMINATION OF I, (*a*) DIFFERENT PORTIONS OF THE SAME SAMPLE BY THE SAME PERSON, (*b*) DIFFERENT PORTIONS OF THE SAME SAMPLE BY TWO DIFFERENT PERSONS IN ONE LABORATORY; AND II, DIFFERENT PORTIONS OF THE SAME MILK BY TEN PERSONS IN TEN DIFFERENT LABORATORIES

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In those countries where legislation for the control of milk exists it has been usual to judge its quality and to assign it to various grades according to the number of colonies of micro-organisms determined by the plate count method. It has of course been known for many years that the plate count does not, in the majority of cases, reflect the actual numbers of organisms present in the milk but rather the number of clumps of organisms capable of giving rise to visible colonies on plates of that medium which happens to be employed. Similarly the amount of agitation of the sample, the temperature of incubation of the plates and the method of enumerating the colonies, to mention only three of a number of factors, have been recognised as causes of error, but the total experimental errors have in the past usually been the subjects of apology when attacks upon the plate count or perhaps upon the conclusions drawn from it have been made.

In spite of these errors a tradition has arisen that the plate count must be the yard stick by which the suitability or accuracy of other methods of judgment must be measured.

It is clearly necessary that, before a method can safely be used as a standard, its own errors must be known so that the proper allowances may be made and false conclusions avoided. The present paper deals with two co-operative experiments designed to examine personal and other errors in the plate count method as used in *routine* practice in the dairy laboratories working under the auspices of the Ministry of Agriculture and Fisheries. The experiment first dealt with was carried out at Shinfield during the years 1928–32. During this time duplicate portions of a large number of samples of the mixed milk from the herd of the National Institute for Research in Dairying were examined by each of two persons.

[1] Dr J. O. Irwin was responsible for the statistical analyses in this paper.

In the second experiment examinations by the same technique were made by ten persons in ten different laboratories. The portions which they examined were derived from a common bulk sample on each occasion, and care was always taken to secure, as far as possible, homogeneity of sampling. Of each set of ten determinations in different laboratories the portion examined at Shinfield constituted one. One of the portions examined at Shinfield was included in the data dealt with in the second part of this paper. The choice was determined by the toss of a coin.

Technique.

The technique employed, for media preparation and for plating, was virtually the same throughout and followed the instructions laid down in the *Guide to the Conduct of Clean Milk Competitions*, pp. 11–13, 3rd edition, Ministry of Agriculture and Fisheries, 1928. All those taking part in the examinations agreed to adhere to this technique categorically and to use the glassware recommended.

Dilutions and counting of colonies.

One plate each of the 1/10, 1/100 and 1/1000 dilution was made for each portion. The numbers on the plates of any series of three dilutions were therefore not averaged, but the first "countable" plate was taken and the number of its colonies multiplied by the appropriate dilution to give the "plate count".

Milk samples.

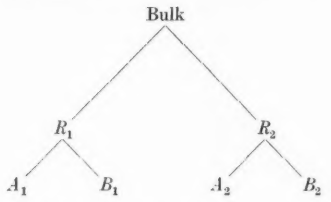
A well-mixed sample of about two quarts of mixed afternoon milk of some 30–50 cows in the herd of the National Institute for Research in Dairying was taken into a sterile Winchester quart bottle and shaken thoroughly. This milk was produced with all the precautions usual on a farm producing certified milk. Eleven portions were then poured into identical six-ounce sterile bottles fitted with rubber stoppers. Each bottle was filled to the same level, packed in corrugated cardboard in a cardboard box and sent by post. Of the eleven portions, two were posted to the National Institute for Research in Dairying and were called R_1 and R_2 respectively. At 4 p.m. on the day following that on which the milk was taken from the cows the portion received by post in each laboratory taking part in the experiment was tested by the agreed technique. If the portions arrived in the laboratory before 4 p.m. they were stored until tested in the 60° F. incubators with which each laboratory was equipped.

Portions R_1 and R_2 provided the material for the "Reading Duplicates".

PART I. THE READING DUPLICATES.

For the purpose of the statistical analysis which follows no plate count of more than 300,000 per 1 ml. was included, since it was considered that overcrowding and counting errors would largely invalidate comparisons between one plate count and another.

From *each* of the portions R_1 and R_2 derived from the original bulk sample and received through the post, two plate counts were made, *i.e.* four examinations of the original milk were carried out, thus:



Bulk = original two-quart sample.
R_1 = first six-ounce portion.
R_2 = second six-ounce portion.
A_1 = examination by observer *A* of portion R_1.
A_2 = examination by observer *A* of portion R_2.
B_1 = examination by observer *B* of portion R_1.
B_2 = examination by observer *B* of portion R_2.

It must be emphasised that *A* or *B* does not necessarily represent the same person throughout the experiment, but *A* and *B* always represented different observers. It is perhaps of some importance that the observers in the Reading laboratory were trained in the details of technique as far as possible in exactly the same way, and no person who was not really skilled was allowed to take part in the experiment. This resulted in a certain uniformity of performance which is exemplified by the fact, that when *all* the plates made from thirty-one samples, of which three portions were examined by one observer and two by another, were counted by each person, the errors due to differences of colony recognition were small.

(1) *Statistical analysis.*

Briefly then the data consisted of a number of colony counts made on plates of 1/10, 1/100 or 1/1000 dilution, each portion of milk being examined in duplicate by two different persons, so that the figures were recorded in groups of four.

For the purpose of this analysis:

(i) In any group of four platings the figures for one dilution only were used.

(ii) In any group of four determinations where the final plate count had been compiled in three cases from the plate of one dilution and in the fourth case from another dilution, that dilution containing the three cases was used in all four cases.

(iii) In any group of four determinations where the final computation of plate count had been made in two cases from one dilution and in two cases from another dilution, the figures for that dilution which was the highest was used in all four cases. It was decided to analyse the data by the "Analysis of Variance Method".

(2) *Choice of unit.*

Some discussion of the correct unit to use for the analysis is necessary. The analysis of variance is strictly speaking applicable only when the variance of the replicates (here sets of four measurements) is independent of the mean (here the mean of the four measurements). Now assuming the bacteria to be uniformly distributed throughout the dilution, and the technique to be ideal, the frequency of the number of colonies on parallel plates should follow the Poisson series. It is a property of the Poisson series that the variance of the measured quantity (here the number of colonies) should be equal to the mean, from which it follows that the variance of the square root of the number of colonies is, in such a distribution, independent of the mean. *A priori*, it therefore seemed that the square root of the colony number would be the correct unit to use. This unit was accordingly adopted and the analysis performed first for the 1/10 dilution. It was found in actual fact that the standard deviations and means of the square roots of the colony numbers in the same sample were *not* uncorrelated, but had a significant correlation of 0·48 based on 145 observations. The average increase in the estimated standard deviation for unit increase in the mean was found to be 0·11 unit. The ideal conditions were thus *not* satisfied, which may be interpreted as meaning that the error of the determination on the plates with the greater number of colonies was relatively greater than would be expected on the ideal hypothesis, perhaps because errors of counting are greater on the more crowded plates. It thus became desirable to try and find a unit which would eliminate the correlation between mean and variance, and the logarithm of the count seemed naturally to suggest itself. When the logarithm of the count was used as a unit the correlation between mean and standard deviation for the 1/10 dilution was found to be $-0{\cdot}16$, a small but probably significant value. There was a suggestion here of a slight over-correction, but the correlation was so small as not to effect the analysis materially.[1]

For safety the analysis was performed three times, with the colony number as unit, with the square root as unit and with the logarithm as unit; the

[1] At the 1/100 dilution using log values the correlation worked out at $-0{\cdot}43$ which is certainly significant for 60 observations, at 1/1000 to $-0{\cdot}39$ which would only occur about once in a hundred times by chance, so that there is little doubt that the logarithm over-corrects somewhat in each case. The ideal unit to use would be some positive power of the colony number less than $\frac{1}{2}$, and could be found by trial and error. This would, however, not be worth doing. We shall see from the appendix that in practice it makes no essential difference to our conclusions whether we use the logarithm or the square root.

results on the last two bases were found not to differ in any essential particular, the same conclusions being drawn in each case.[1]

(3) *Method of analysis.*

The essential principle of the method is that the sum of the squares of the deviations of all our observations from the mean may be divided up into a number of parts or items, each of which corresponds to a particular source of variation. Were the data completely homogeneous then on dividing each item by an appropriate divisor we should obtain an estimate of the same variance, namely the variance of the universe of which the homogeneous data was a sample. These divisors are known as "degrees of freedom", because they are equal to the number of independent squares to which the corresponding items are equivalent. In actual practice we find that some of our estimates of variance turn out to be greater than others. This may be due to sampling errors only, or it may be because some of the sources of variation really are greater than others, and a test exists for determining whether any two items differ significantly, that is to say, whether their difference is greater than would arise from random sampling.[2] The method thus enables us to find the important sources of variation and experimental error.

Table I. 145 *samples, four plate counts of each: total* 580.

1/10 dilution. Log units.

Variation due to differences	Analysis of variance: Sum of squares		Degrees of freedom		Mean squares	
(1) Between samples	69·2467		144		0·4809	
(2) *A v. B* (observers)	0·0022		1		0·0022	
(3) Between duplicates	0·0143		1	3	0·0143	
(4) Interaction between (2) and (3)	0·0091		1		0·0091	
(5) Error:						
(6) Interaction between (1) and (2)	2·3624		144		0·0164	
(7) Interaction between (1) and (3)	3·0828	7·8553	144	432	0·0214	0·0182
(8) Interaction between (1) and (4)	2·4101		144		0·0167	
Total	77·1276		579			

Table I shows the analysis for the 1/10 dilution (the analysis being based on the logarithms of the colony numbers). The clue to the form of the analysis

[1] It will be noted in the appendix that the percentage which the experimental error is of the mean *does* depend on the unit used as the basis of the analysis. But as long as we keep to the same unit, this percentage remains practically constant at all three dilutions. Thus the change in the percentage when the unit is changed means that we are presenting the same facts in a different way rather than that we are arriving at different conclusions.

[2] The test consists in calculating $z = \frac{1}{2} \log \frac{v_1}{v_2}$, where v_1, v_2 are two estimates of variance based respectively on n_1, n_2 degrees of freedom ($n_1 > n_2$). We then refer to a table which gives the 5 and 1 per cent. points of the distribution of z, *i.e.* the values such that values as large or larger would only occur by chance respectively once in twenty or once in one hundred times. If z is greater than its 5 per cent. point we regard the difference as significant. The 5 per cent. point may be used if a more stringent test is required.

is provided by a consideration of the degrees of freedom. Thus at the 1/10 dilution, there were 145 samples and four counts on each sample, giving 580 observations. This gives us 579 degrees of freedom. Of these 144 degrees of freedom (item (1)) are due to differences between the 145 samples as a whole, 435 (3×145) are due to differences within samples. Of the latter there will be 3 ($4-1$) due to differences between the four subcounts as a whole. One of these (item (2)) corresponds to differences between A's results as a whole and B's results as a whole, *i.e.* measures any tendency to a systematic difference between A's and B's results. Another (item (3)) corresponds to the differences between duplicates as a whole, while the third (item (4)) corresponds to the interaction between the former two, *i.e.* any tendency for a systematic difference between A's and B's results to be different in one duplicate from the other.

There remain 432 (3×144) degrees of freedom due to any tendency of the three items just mentioned to differ in relative importance from sample to sample, which may briefly be referred to as "error" (item (5)), but it may be noticed that this "error" may itself be split up into three portions, 144 due to interaction between (1) and (2), *i.e.* to any tendency of the difference between A's and B's results to vary from sample to sample (item (6)), 144 due to interaction between (1) and (3), *i.e.* to any tendency of the difference between duplicates to vary from sample to sample (item (7)), and finally 144 due to interaction between (1) and (4), *i.e.* any tendency of the effect (4) to vary from sample to sample (item (8)).

It will, of course, be noticed that the degrees of freedom and the corresponding items in the "sum of squares column" are additive. The mean square column in item (6) is in fact an estimate of the variance of the mean difference between A's and B's results from sample to sample; that in item (7) is an estimate of the variance of the mean difference between duplicates from sample to sample. These two items clearly have considerable importance.

The advantage of the "analysis of variance method" is that the data are considered comprehensively, and each source of variation is considered in its proper place.

A consideration of Table I shows, as would naturally be expected, that the most important source of variation is the heterogeneity of the samples themselves. The remaining items due to personal and experimental error are all of the same order of magnitude. (There is a suggestion indeed that item (2) is smaller than the others, but as it is based on only one degree of freedom we cannot assert the difference to be significant. Comparing items (2) and (5) gives $z = 0{\cdot}70$ with a 5 per cent. point of 2·77.)

In other words all the sources of variation contribute about the same amount to the experimental error, and we may combine items (2), (3), (4) and (5) and obtain the figure $7{\cdot}881/435 = 0{\cdot}0181$ as the variance due to experimental error. This corresponds to a standard deviation of 0·135, which is 7·6 per cent. of the mean. This may be used as a basis for the calculation of

standard errors, for example the mean of the A_1 log counts is 1·77 with a standard error of $\sqrt{\frac{0{\cdot}0181}{145}} = 0{\cdot}012$. The standard error of the mean count in any sample is $\sqrt{\frac{0{\cdot}0181}{4}} = 0{\cdot}067$. Any two samples may be judged to differ significantly if their mean log counts differ by more than 2·8 or roughly 3 times this, *i.e.* about 0·2. We have:

Mean log counts.

A_1	A_2	B_1	B_2	Standard error	General mean	Standard error
1·77	1·79	1·78	1·78	0·012	1·78	0·006

The estimated standard deviation of the differences, in each sample, between A's and B's mean results is $\sqrt{0{\cdot}0164}$ or 0·129, that between duplicates is $\sqrt{0{\cdot}0214} = 0{\cdot}146$. These do not differ significantly, in fact the estimate $\sqrt{0{\cdot}0181} = 0{\cdot}135$ can be used for both. We may deduce from this experimental error of 0·135 that 95 per cent. of counts in the same sample will lie between 1·9 times the mean and 1/(1·9) of the mean.

To sum up, at a dilution of 1/10, the experimental error is about 8 per cent. of the mean. All the sources of variation contribute about equal amounts to this, and there is no evidence that there is more variation in the differences between two plate counts when tested by two different people than when tested by the same person in this laboratory.

Table II. *Sixty samples, four plate counts of each: total* 240.

1/100 dilution. Log units.

Variation due to differences	Analysis of variance: Sum of squares		Degrees of freedom		Mean squares	
(1) Between samples	14·3372		59		0·2430	
(2) *A v. B* (observers)	0·1347		1	3	0·1347	
(3) Between duplicates	0·0194		1		0·0194	
(4) Interaction between (2) and (3)	0·0017		1		0·0017	
(5) Error:						
(6) Interaction between (1) and (2)	1·7178	4·8255	59	176*	0·0291	0·0274
(7) Interaction between (1) and (3)	1·6993		59		0·0288	
(8) Interaction between (1) and (4)	1·4084		*58		0·0243	
Total	19·3185		*238		0·0812	

* One observation was missing out of the 240. This was accordingly assumed equal to its duplicate, and one degree of freedom deducted from those due to error.

Turning to dilution 1/100 Table II shows the analysis of variance. The most striking feature here is the relatively large item due to the systematic difference between A's and B's results. Item (2) is in fact significantly greater than item (5). ($z = 0{\cdot}79$ with a 5 per cent. point of 0·68.) Thus there is here, for some reason, a significant systematic difference between A's and B's results. The variance due to experimental error will, apart from this source

of variation, be 0·0272, corresponding to a standard deviation of 0·165 or 8·7 per cent. of the mean. The standard error of the mean of a single sample is thus $\sqrt{\frac{0{\cdot}0272}{4}} = 0{\cdot}082$, so that any two samples where the mean results differ by more than 0·25 may be regarded as significantly different. Thus two samples differ significantly when the ratio of their geometric mean counts is greater than 1·8. The corresponding figure for the 1/10 dilution is 1·6.

For the 1/100 dilution we have:

Mean log counts.

A_1	A_2	B_1	B_2	Standard error	General mean	Standard error
1·86	1·88	1·91	1·92	0·021	1·89	0·010

The difference between the means of A's and B's results is 0·045 with a standard error of 0·021, which confirms the significance of the systematic difference already found.

The estimated standard deviation of the differences, in each sample, between A's and B's mean results is $\sqrt{0{\cdot}0291} = 0{\cdot}171$, that between duplicates is $\sqrt{0{\cdot}0288} = 0{\cdot}170$. These do not differ significantly, and the estimates $\sqrt{0{\cdot}0272} = 0{\cdot}165$ may be used for both. 95 per cent. of counts in the same sample will lie between 2·2 times the mean and 1/(2·2) of the mean.

The importance of the systematic difference between A's and B's results may be estimated in the following way. The total variance may, in the light of the analysis, be conceived as being made up of three portions.

	Variance
(1) Due to differences between samples	$\frac{0{\cdot}2430 - 0{\cdot}0272}{4} = 0{\cdot}0539$
(2) Due to systematic differences between A's and B's results	$\frac{0{\cdot}1347 - 0{\cdot}0272}{120} = 0{\cdot}0009$
(3) Due to remaining experimental error	$0{\cdot}0272 = 0{\cdot}0272$
Total	0·0820*

* The difference between this result and the figure 0·0812 in Table II, is due to a small bias inherent in the former estimate.

The systematic difference thus accounts for only 1 per cent. of the total variance.

Summing up the results for the 1/100 dilution, there is a systematic difference between A's and B's results whose average effect is quite small. This effect is of course eliminated in comparing the average results of samples examined by both A and B. Apart from it the experimental error is 8·7 per cent. of the mean. In spite of the existence of the systematic error there is no evidence that the difference between A's and B's results varies more from sample to sample than the difference between two counts made by the same person.

Table III. *Thirty-seven samples, four plate counts of each: total* 148.

1/1000 dilution. Log units.

Variation due to differences	Analysis of variance: Sum of squares		Degrees of freedom		Mean squares	
(1) Between samples	40·5024		36		1·1251	
(2) *A v. B* (observers)	0·0412		1	3	0·0412	
(3) Between duplicates	0·0040		1		0·0040	
(4) Interaction between (2) and (3)	0·0003		1		0·0003	
(5) Error:						
(6) Interaction between (1) and (2)	0·2702	1·8008	36	108	0·0075	0·0167
(7) Interaction between (1) and (3)	0·7337		36		0·0204	
(8) Interaction between (1) and (4)	0·7969		36		0·0221	
Total	42·3487		147		0·2881	

Table III shows the analysis for the 1/1000 dilution. Item (6) appears to be significantly lower than (7) or (8), but it is difficult to see the reason for this. Otherwise the variation due to all sources of error is of the same order. Combining items (2) to (5) we reach the figure of 0·0166 as the variance due to experimental error. This corresponds to a standard deviation of 0·129 or 5·9 per cent. of the mean. The standard error of the mean result of a single sample is therefore 0·064, and two samples where the mean results differ by more than 0·19 will be significantly different. This corresponds to a ratio between the counts of the two samples of about 1·5. For the 1/1000 dilution we have:

Mean log counts.

A_1	A_2	B_1	B_2	Standard error	General mean	Standard error
2·17	2·18	2·20	2·21	0·02	2·19	0·01

95 per cent. of counts in the same sample will lie between 1/(1·8) of the mean and 1·8 times the mean.

Summary.

(1) It has been shown that the logarithm of the colony number is the most appropriate unit to use for analysing this data.

(2) At the 1/100 dilution there is a systematic difference between *A*'s and *B*'s results, the average effect of which is small and which only accounts for 1 per cent. of the variance of a single plate. This difference does not affect the comparison between the sample means, since each is the average of two of *A*'s and of two of *B*'s results.

(3) Apart from this effect, which occurs only at the 1/100 dilution, all sources of error contribute about the same amount to the experimental error which is in each case between 6 and 9 per cent. of the mean (with the units used).

(4) At the 1/10 dilution we can assert two samples to be significantly different when the ratio of their geometric mean colony numbers is greater than 1·6; the corresponding figures are 1·8 at 1/100 and 1·5 at 1/1000. Thus in terms of colony number the three dilutions are equally accurate, but in

terms of "number of bacteria per ml." the 1/10 dilution is roughly ten times as accurate as the 1/100 and the 1/100 ten times as accurate as the 1/1000. For example, taking 100 colonies in each case, whereas at the 1/10 dilution we could just distinguish between results of 1000 bacteria per ml. and 1600 per ml., at the 1/100 dilution we could only just distinguish between 10,000 and 18,000 and at 1/1000 between 100,000 and 150,000. This is a different state of affairs from that which would hold if the "ideal" Poisson hypothesis were correct. For if m were the number of bacteria per ml. the standard error of the count obtained from a single plate would at the three dilutions be respectively $\sqrt{10m}$, $\sqrt{100m}$, $\sqrt{1000m}$, and the accuracy would decrease as the square root of the dilution number instead of, as in practice appears to be the case, the dilution number itself. Thus not only, as we pointed out in the text, is the accuracy of the more crowded plates at any one dilution less than would be expected on the "ideal" Poisson hypothesis, but the accuracy falls off more rapidly with increasing dilutions than would be expected on that hypothesis.

(5) At dilution 1/10, 95 per cent. of counts in the same sample would lie between about 1·9 times the mean and 1/(1·9) of the mean. The corresponding figures for the 1/100, 1/1000 dilutions are 2·2 and 1·8 respectively; that is to say, the ratio of the upper to the lower limit, where a large number of counts are made, would be about 4 to 1.

Appendix.

In order to examine the effect of the unit chosen on the analysis of variance, the analyses were in each case performed on

(1) the colony numbers themselves,
(2) the square roots of the colony numbers,
(3) the logarithms of the colony numbers.

At the 1/10 dilution there was a correlation of 0·70 between the mean and standard deviation of a sample, so that the colony numbers were clearly not a good unit to use, nevertheless it seemed of interest to know how much the correlation would affect the analysis. Tables IV, V and VI show for dilution 1/10, 1/100 and 1/1000 the variances due to each item of the analysis; when it is based on

(1) the colony numbers,
(2) the square roots of the colony numbers,
(3) the logarithms of the colony numbers.

The square root units and the logarithm units always show the same significant differences or the same absence of significant differences. On the whole at each dilution the different items maintain the same ratio to one another. If we used the colony numbers as the basis of the analysis we should get a somewhat distorted view of things, but this distortion is smaller than one might have expected considering the magnitude of the correlation (0·7) between the mean and standard deviation of a sample. For example, at the 1/10 dilution,

if we used the original units we should conclude that item (4) was significantly less than items (2), (3) or (5), whereas this is actually not the case (as the use of the more correct logarithmic units tells us). It would also be suggested that item (6) was significantly greater than item (8), which really appears not to be the case. Again at the 1/100 dilution a significant difference would be suggested between items (2) and (5), and between items (6) and (8), but the

Table IV. *Dilution* 1/10. *Analysis of variance on three different bases.*

Variation due to differences	Degrees of freedom	Variance calculated from: Original units		Square root units		Logarithmic units	
(1) Between samples	144	17,332·90		44·81		0·4809	
(2) *A v. B* (observers)	1	1,081·49		1·04		0·0022	
(3) Between duplicates	1	801·94		1·56		0·0143	
(4) Interaction between (2) and (3)	1	0·84		0·16		0·0091	
(5) Error:							
(6) Interaction between (1) and (2)	144	1,160·28		1·67		0·0164	
(7) Interaction between (1) and (3)	144	1,016·47	981·82	2·11	1·78	0·0214	0·0182
(8) Interaction between (1) and (4)	144	768·71		1·54		0·0167	
	432						

Table V. *Dilution* 1/100. *Analysis of variance on three different bases.*

Variation due to differences	Degrees of freedom	Variance calculated from: Original units		Square root units		Logarithmic units	
(1) Between samples	59	11,957		28·74		0·2430	
(2) *A v. B* (observers)	1	4,301		13·55		0·1347	
(3) Between duplicates	1	989		2·48		0·0194	
(4) Interaction between (2) and (3)	1	146		0·17		0·0017	
(5) Error:							
(6) Interaction between (1) and (2)	59	1,237		2·89		0·0291	
(7) Interaction between (1) and (3)	59	1,060	967	2·64	2·32	0·0288	0·0274
(8) Interaction between (1) and (4)	58	597		1·41		0·0243	
	176						

Table VI. *Dilution* 1/1000. *Analysis of variance on three different bases.*

Variation due to differences	Degrees of freedom	Variance calculated from: Original units		Square root units		Logarithmic units	
(1) Between samples	36	1,416,664		514·59		1·1251	
(2) *A v. B* (observers)	1	2,466		6·79		0·0412	
(3) Between duplicates	1	24,034		6·66		0·0040	
(4) Interaction between (2) and (3)	1	21,311		1·74		0·0003	
(5) Error:							
(6) Interaction between (1) and (2)	36	6,732		4·08		0·0075	
(7) Interaction between (1) and (3)	36	17,278	11,897	4·66	3·93	0·0204	0·0167
(8) Interaction between (1) and (4)	36	11,200		3·05		0·0221	
	108						

analysis still shows the systematic difference between *A*'s and *B*'s results. At the 1/1000 dilution significant differences would be suggested between items (6) and (7) and (6) and (8).

Table VII shows us that, although the percentage that the experimental error is of the mean depends on the unit used, nevertheless, whatever unit is used, the percentage remains of the same order at all three dilutions. We are simply presenting the same facts in a different way.

Table VIII shows the standard deviation due to each item, for each kind of unit used, both in absolute units and with (1) as 100. On the whole the same proportions between the different items are obtained. This may be best seen by comparing items (1) and (5). Items (2), (3), (4) are only based on one degree of freedom each, and are consequently subject to very large

Table VII. *Variance and standard deviation due to experimental error. Different bases of calculation compared.*

	General mean	Variance due to experimental error	S.D.
Dilution 1/10:			
Original units	83·93	979	31·3 37·3 %
Square root units	8·45	1·77	1·33 15·7 %
Log units	1·78	0·0181	0·135 7·6 %
Dilution 1/100:			
Original units	95·58	962	31·0 32·4 %
Square root units	9·31	2·29	1·51 16·2 %
Log units	1·89	0·0271	0·165 8·7 %
Dilution 1/1000:			
Original units	362·82	12,005	109·5 30·1 %
Square root units	15·32	3·96	1·99 13·0 %
Log units	2·19	0·0167	0·129 5·9 %

Table VIII. *Standard deviations, due to different sources of variation compared, on three different bases of calculation.*

Dilution	Item	Original units	(1) as 100	Square root units	(1) as 100	Logarithmic units	(1) as 100
1/10	(1)	131·7	100	6·69	100	0·693	100
	(2)	32·9	25·0	1·02	15·2	0·047	6·8
	(3)	28·3	21·5	1·25	18·7	0·120	17·3
	(4)	0·9	0·7	0·40	6·0	0·030	4·3
	(5)	31·3	23·8	1·33	19·9	0·135	19·5
1/100	(1)	109·3	100	5·36	100	0·483	100
	(2)	65·6	60·0	3·68	68·7	0·367	76·0
	(3)	31·4	28·7	1·58	29·5	0·139	28·8
	(4)	12·1	11·1	0·41	7·6	0·041	8·5
	(5)	31·1	28·5	1·52	28·4	0·166	34·4
1/1000	(1)	1190·2	100	22·68	100	1·006	100
	(2)	49·7	4·2	2·61	11·5	0·203	20·1
	(3)	155·0	13·0	2·58	11·4	0·063	6·3
	(4)	146·0	12·3	1·32	5·8	0·017	1·7
	(5)	109·1	9·2	1·98	8·7	0·129	12·8

sampling fluctuations to which may be attributed the irregularities observed. The most striking departure from proportionality is exhibited by item (2) at the 1/10 dilution; this is 25 per cent. of item (1) when the original units are used, 15 per cent. of item (1) when square root units are used, and 7 per cent. when logarithmic units are used. This steady decline is almost certainly an accident, for items (2) and (3) do not differ significantly whatever the basis

of the calculation. To save complication item (5) has not here been split up into its component portions.

The general conclusion seems to be that a small or moderate correlation between the mean and standard deviation of replicates does not affect the analysis of variance materially. Even a moderately large correlation (0·7) affects the analysis only to a moderate extent. While this effect is perhaps less than might have been anticipated, it is nevertheless rather misleading.

Part II. The duplicate testing scheme.

A general description of the source of the portions of milk used to secure the data analysed below has been given earlier in this paper, but a rather more detailed statement may be advantageous.

The original sample was divided as described and the portions dispatched by post to the ten laboratories (stations) taking part in the experiment. Thus:

Sample → R_1, R_2 } Reading (see text)
C
D
E
F
G
H
I
J
K

It must be emphasised that the portions were sent by post without precautions for controlling temperature and agitation, *i.e.* all portions were subject to the general hazards of transit. All portions were, however, examined at the same age (about 24 hours) by an agreed technique on media of the same composition.

Method of analysis.

In analysing the figures, it was decided to use the count for that dilution which would ordinarily be used in practice in reporting the count. As a basis for the analysis, the logarithms of these counts were used, previous work on the Reading Duplicates (Part I) and by other workers (for instance Robertson and Fryer(1)) having shown that the logarithm was the most reasonable unit to use.

419 samples were available and should have yielded 4190 counts; actually there were 412 counts missing, and the method of analysis involving an estimate being made of these, the geometric mean of the available counts in the same sample was in each case used for the missing count. In calculating the experimental error an allowance was made for this adjustment.

In analysing the results, the "analysis of variance" method was again used. Here, the sum of the squares of the deviations of all the observations from the mean can be shown to be composed of three portions:

(1) Ten times the sum of the squares of the deviations of the sample means from the general mean.

(2) 419 times the sum of the squares of the deviations of station means from the general mean.

(3) A residual portion which would vanish if the deviation of any station's log-count from the mean of the sample were the same for all samples.

On dividing the portions (1), (2), (3) by the appropriate number of "degrees of freedom" we obtain three items: (1) ten times the square of the standard deviation of the sample means, (2) 419 times the square of the standard deviation of the station means, and (3) an item due to differences in the relative behaviour of stations from sample to sample. The latter may reasonably be ascribed to experimental error.

Another way of looking at the same thing is to suppose that each log-count (x) is composed of two independent portions (x_s and x_o) dependent respectively on the sample to which the count belongs and the station who made it, or rather on the general conditions under which it was made. We should then have

$$x = x_s + x_o.$$

Owing, however, to experimental error this will not be true exactly for each count, and the best values of x_s and x_o can be determined by the method of least squares. The best value of x_s turns out to be the mean of the ten counts in the sample "s", that of x_o to be the mean of the 419 counts in the station "o", and the square of the standard deviation of $x - x_s - x_o$ is then item (3) above, which is due to experimental error.

Were the data completely homogeneous the items (1), (2) and (3) would all give estimates of the same variance, namely, the variance of the universe of which the data are samples. As it is, we shall find items (1) and (2) are as a rule significantly greater than item (3), in other words that there are significant differences between different samples and different stations, and we shall be able to estimate the amount of variation due (1) to differences between samples, (2) to differences between stations, and (3) due to experimental error.

(i) The results for the 4190 counts are as follows:

Table I.

(Unit the logarithm of the count.)

	Sum of squares	Degrees of freedom	Mean squares	S.D.	
(1) Between samples	1351·96	418	3·234	1·80	
(2) Between stations	30·09	9	3·343	1·83	0·48
(3) Error	733·35	3350*	0·219	0·47	
Total	2115·40	3777*	0·560	0·75	

* 3350 = 3762 − 412; 3777 = 4189 − 412, 412 is the number of missing counts; this is the allowance made for the adjustment mentioned above.

It will be observed that there are systematic differences between stations, the mean square 3·34 between stations being significantly greater than the mean square 0·22 due to error ($z = 1{\cdot}36$, 1 per cent. point $= 0{\cdot}44$).

The mean results for the different stations and their standard error are given in Table A. Two mean counts whose logs differ by more than three times

the standard error may be regarded as significantly different, *i.e.* two mean counts (taken at random) whose ratio is greater than 1·17 are significantly different.

To obtain the average variance within samples we must combine items (2) and (3). This gives 0·24 or a standard deviation of 0·48. That is in 95 per cent. of counts in the same sample the log-count will be within 0·96 of the mean, or actual counts may range from one-ninth of the mean to nine times the mean. In 80 per cent. of samples the counts will be within 1/(4·1) of the mean and 4·1 times the mean.

The variance of the whole 4190 counts is 0·560 or their standard deviation 0·75, we may estimate how much of this is due (1) to real sample differences, (2) to systematic station differences, and (3) to experimental error.

		Variance	S.D.
(1) Sample differences	$\frac{3·234 - 0·219}{10}$	0·302	0·55
(2) Station differences	$\frac{3·343 - 0·219}{419}$	0·007	0·08
(3) Experimental error		0·219	0·47
		0·528*	

* The difference between the total 0·528 and the figure 0·560 in Table I is mostly due to the effect of estimating the 412 missing counts, and partly to a small bias inherent in the latter estimate. It will be seen later that in cases where only the latter factor operates there is agreement to two decimal places.

(ii) Since in the whole data 412 counts were missing, it was thought advisable to examine separately the 129 samples which were complete, *i.e.* in which a count had been recorded for every station.

These yielded the following results:

Table II. *Analysis of variance.*

(Unit the logarithm of the count.)

	Sum of squares	Degrees of freedom	Mean squares	S.D.	
(1) Between samples	581·17	128	4·540	2·13	
(2) Between stations	12·89	9	1·432	1·20	0·48
(3) Error	260·21	1152	0·226	0·48	
Total	854·27	1289	0·662	0·81	

Here the differences between samples are somewhat greater than before; experimental error is the same.

The mean results for the different stations and their standard error are given in Table A.

The coefficient of correlation between the two series of mean counts, provided (α) by all the 419 samples and (β) by the 129 complete samples, is 0·91. There is therefore little doubt that both series present substantially the same picture of the systematic differences.

The average variance within samples is 0·24, giving a standard deviation of 0·48 as before, and as before in 95 per cent. of counts in the same sample

the log-count will be within 0·96 of the mean, or actual counts may range from one-ninth of the mean to nine times the mean. In 80 per cent. of counts in the same sample the counts will be within 1/(4·1) of the mean and 4·1 times the mean.

The contributions of the sample differences, the systematic station differences and the experimental error to the total variance are again given in Table B. The total variance is 0·666 against 0·662 given by the direct calculation of Table II.

Table A. *Station mean counts and their standard errors.*

	(1) All 419 samples		(2) The 129 complete samples		(3) 31 samples all at dilution 1/10	
Station	Logarithm	Geometric mean	Logarithm	Geometric mean	Logarithm	Geometric mean
B_2	3·3033	2010	3·3924	2468	2·6645	462
C	3·3761	2377	3·4122	2584	2·6535	450
D	3·3342	2159	3·3871	2438	2·8064	640
E	3·1418	1386	3·1905	1550	2·7310	538
F	3·3020	2004	3·4039	2534	2·7200	525
G	3·1404	1382	3·2006	1587	2·6032	401
H	3·2109	1625	3·2124	1631	2·6197	417
I	3·3745	2369	3·4163	2608	2·7048	507
J	3·1860	1535	3·2067	1609	2·6671	465
K	3·2677	1852	3·4204	2633	2·7445	555
	S.E. (0·0229)	antilog (3 × S.E.) = 1·17	S.E. (0·0418)	antilog (3 × S.E.) = 1·33	S.E. (0·0503)	antilog (3 × S.E.) = 1·415
	Two mean counts, taken at random, whose ratio is greater than 1·17 are significantly different		Two mean counts, taken at random, whose ratio is greater than 1·33 are significantly different		Two mean counts, taken at random, whose ratio is greater than 1·415 are significantly different	

Table B. *Showing proportions of total variance, due respectively to sample differences, systematic station differences and experimental error.*

	All 419 samples		129 complete samples		31 samples, all counts at 1/10	
	Variance	S.D.	Variance	S.D.	Variance	S.D.
Sample differences	0·302	0·55	0·431	0·66	0·0162	0·13
Station differences	0·007	0·08	0·009	0·09	0	0
Experimental error	0·219	0·47	0·226	0·48	0·0796	0·28
Total	0·528	0·73	0·666	0·82	0·0958	0·31

(iii) In order to investigate the influence of using different dilutions at different stations on the systematic station differences, thirty-one complete samples in which all counts were made at a dilution of 1/10 were analysed separately. The results are as follows:

Table III. *Analysis of variance. Dilution* 1/10.

(Unit the logarithm of the count.)

	Sum of squares	Degrees of freedom	Mean squares		S.D.	
(1) Between samples	7·2626	30	0·2421		0·482	
(2) Between stations	1·0628	9	0·1181	0·0796	0·334	0·282
(3) Error	21·1564	270	0·0784		0·280	
Total	29·4818	309	0·0954		0·309	

Here the variation is much smaller, item (2) is no longer significantly larger than item (3), but (1) is significantly larger than (2) and (3) combined.

Thus there are no systematic differences between stations, and combining (2) and (3) gives the average variance within samples, or the variance due to experimental error. This is 0·0796, corresponding to a standard deviation of 0·282; thus in 95 per cent. of counts in the same sample the log-count will be within 0·564 of the mean, or actual counts may range from 1/(3·7) of the mean to 3·7 times the mean.

In 80 per cent. of counts in the same sample the counts will be between 1/(2·3) of the mean and 2·3 times the mean.

The mean results for the different stations and their standard errors are given in Table A; it will be observed that the means do not differ significantly.

The contribution of sample differences and experimental error to the total variance is as before given in Table B. It will be observed that the total only differs by 0·0004 from the direct calculation of Table III.

Since when all the counts are at the same dilution, there are no systematic differences between stations, it is probable that the systematic station differences exhibited by the 419 samples and the 129 complete samples are due to the use of plates at different dilutions in the different stations. Now those plates were selected (for calculating the count) which contained between 30 and 300 colonies, hence the use of plates at different dilutions may be taken as indicating underlying differences in the conditions of the samples at the different stations to which the systematic differences are really due.

The systematic differences being apparently due to conditions of temperature over which the observers themselves have no control, it would seem of interest to enquire into the further problem of whether the observers themselves are equally accurate.

Summary.

(1) 419 samples were counted in each of ten stations (laboratories) in order to assess the accuracy of the plate-count method under the conditions existing in practice.

(2) When, in each count, that plate which would ordinarily be used in practice is selected for the final count, it is found that the range which would include 95 per cent. of counts in the same sample is as wide as from one-ninth of the mean to nine times the mean when inter-station differences are taken into account. For 80 per cent. of counts in the same sample the corresponding range would be from 1/(4·1) of the mean to 4·1 times the mean.

(3) Systematic differences between stations under these conditions account for between 3 and 4 per cent. of the total experimental variation.

(4) When thirty-one complete samples in which all counts were made at a dilution of 1/10 were statistically analysed separately it was found that the range which would include 95 per cent. of counts in the same sample was from 1/(3·7) of the mean to 3·7 times the mean. For 80 per cent. of counts in

the same sample the corresponding range would be from 1/(2·3) of the mean to 2·3 times the mean. There were no systematic station differences.

(5) When all plates are counted at the same dilution the error is therefore much reduced and this result suggests that the systematic station differences exhibited by the 419 samples as well as some of the random errors occurring in them is due to the combining of plate counts made at different dilutions.

In effect this means that owing to the different conditions encountered during transit comparable figures for any sample could not be expected.

Conclusions.

Owing to the varying conditions of temperature and perhaps agitation encountered by milk dispatched through the post to various parts of the country and to the personal errors of technique amongst observers at different laboratories—although they were nominally following exactly the same technique—it seems that the practice of making comparisons between the results of plate counts on the same milk but carried out in different laboratories should be abandoned if no precautions to secure uniform conditions of transit are taken.

Conclusions.

It may be concluded from the statistical analysis of these data that:

(*a*) When two observers examine the same sample of milk by the technique described the differences in the results are not greater than when one worker examines the same sample in duplicate. This is probably only true when, as in this case, the observers are trained to the same standard.

(*b*) When different individual samples of milk are examined by two workers whose performances are nearly identical it is necessary to allow a wide margin before the differences between their results can be asserted to be truly significant.

(*c*) Owing to the wide differences in the results secured by the examination of portions of the same milk by different observers in different laboratories, it is clear that the practice of comparing results from one laboratory with those of another must be abandoned.

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101. THE OCCURRENCE OF COLIFORM ORGANISMS IN CREAM AND THEIR EFFECT UPON CREAM QUALITY[1]

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The methods of producing and handling cream for butter-making in New South Wales are such that coliform organisms are found abundantly in this product; little is known, however, regarding the incidence of specific types within the group. It is hardly possible to base an assumption in this respect upon the results of investigations into their occurrence in milk and other dairy products, as the factors influencing bacterial development are not comparable. The subject has been most fully investigated by Yale(1), who, in a study of the group in dairy products, isolated forty-two cultures from twenty-four samples of raw and sour cream and found that 33 per cent. were of the true *coli* type, 57 per cent. *aerogenes*, and the remainder intermediate types. In the following section are presented the results of an analysis of the *coli-aerogenes* organisms isolated from 112 samples of commercial cream at a butter factory in New South Wales.

Preliminary isolations.

Samples were taken from the cream deliveries of 112 individual suppliers on the platform of the Tumut Butter Factory, New South Wales, in November 1933. In order to secure as wide and diverse a range as possible only one sample was taken from each supplier's cream. The age of the cream varied from 4 to 48 hours. In taking samples no discrimination was exercised either in the age or the grade of the cream.

From each can approximately 1/100 ml. of cream was transferred by means of a sterile platinum loop to tubes of lactose peptone broth containing 0·001 per cent. gentian violet. It was not possible to incubate the tubes until return to the laboratory, and they were of necessity held for 72 hours at climatic temperatures which ranged between 20 and 30° C. At the end of this time a loop from each tube was transferred to fresh fermentation tubes which were incubated for 48 hours at 37° C.

Streak cultures were made from each tube showing positive results (including the original inoculations) on eosin-methylene-blue agar and the plates were incubated for 48 hours at 37° C. They were then examined under a

[1] This work was carried out by the author during his tenure of the Sydney University Walter and Eliza Hall Agricultural Research Fellowship.

binocular microscope, and each colony revealing individual colour and structural characteristics on a plate was fished and transferred to lactose broth fermentation tubes. After 48 hours' incubation at 37° C. the re-isolated gas-forming cultures were again streaked and the plates incubated at 37° C. Where it was evident from the colony types on the plates that mixed strains were being cultured, they were repeatedly grown in lactose broth and restreaked on various media (eosin-methylene-blue agar, Endo's agar, and the brilliant green-citrate agar advised by Kon(2)) until apparently pure strains were secured.

DIFFERENTIAL TESTS.

The citric acid fermentation test, the Voges-Proskauer reaction, the methyl-red test, and the indol test were used to differentiate *Bact. coli* from *Bact. aerogenes*. Where possible the procedures advised in the *Standard Methods for Water Analysis*, 1925, and also the *Manual of Methods for the Pure Culture Study of Bacteria*, were followed. In the case of the indol test, organisms were grown in nutrient broth containing Witte's peptone and 0·01 per cent. tryptophane for 24 hours at 37° C. The formation of indol was shown with Kovac's(3) modification of Ehrlich's indol reagent which gave very satisfactory results.

Strains were tested for acid and gas production in sucrose, salicin and dulcitol. Organisms giving positive reactions for *Bact. aerogenes* were further tested for gelatin liquefaction and the fermentation of glycerol and starch. Evidence of motility was based upon the examination in a hanging drop of cultures in nutrient broth held at 30° C. for 24 hours.

Results.

Coliform bacteria were isolated from eighty-eight (or 72 per cent.) of the 112 samples of cream examined. Organisms from an individual sample giving similar fermentative and other reactions were assumed to be identical, and after their elimination 128 cultures were obtained. Of these ninety-eight (76·5 per cent.) gave positive reactions for *Bact. coli* and fourteen (11 per cent.) for *Bact. aerogenes*, while sixteen (12·5 per cent.) were intermediate.

The 128 strains were arranged according to the classification suggested by Levine(4). The proportions of the various specific types present may be seen in Table I.

Table I.

Group	Species	Number	% of total	% of total
Coli	*Bact. coli*	44	34·4	76·5
	Bact. communior	35	27·3	
	Bact. acidi-lactici	12	9·4	
	Bact. neapolitanus	5	3·9	
	Bact. coscoroba	2	1·6	
Aerogenes	*Bact. aerogenes*	10	7·8	11·0
	Bact. cloacae	4	3·2	
Intermediate		16	12·5	12·5

Discussion.

Ruchhoft, Kallas, Chinn and Coulter(5) have shown that preliminary enrichment methods of isolation of coliform organisms may not give a correct impression of original conditions owing to the tendency of one organism to outgrow the other. The results therefore must be interpreted in the light of their findings. There is a suggestion in their work, however, that the development of *Bact. aerogenes* is favoured under these circumstances, and as in the present case the temperatures of preliminary enrichment were low and would presumably also favour the growth of *Bact. aerogenes*, it is considered that the high proportion (76·5 per cent.) of typical *Bact. coli* forms isolated has some significance.

A comparison of the fermentation and other reactions of the *Bact. coli* type organisms isolated from cream, with the characteristics of coliform bacteria in bovine faeces, would confirm the natural supposition that these organisms are derived from the latter source. The following figures are taken from a table given by Rogers, Clark and Lubs(6), showing the percentage of positive acid and gas reactions in low ratio coliform organisms isolated from various sources. They are compared with the results in the present experiment.

Table II.

Group	Source	Number	% indol	% sucrose	% dulcitol
Low ratio (*coli*)	Human faeces	131	96·95	17·56	31·78
	Bovine faeces	149	99·30	59·10	68·20
	Cream	98	100·00	44·00	67·00

Similarly on the basis of the classification suggested by Levine(4) it will be seen that *Bact. coli* and *Bact. communior* predominate amongst the low ratio organisms isolated from cream, comprising 78 per cent. of the total. Levine states that these organisms occurred most frequently in bovine faeces, comprising 75 per cent. of the total.

High ratio or *aerogenes* type organisms are found infrequently in normal bovine faeces. Rogers, Clark and Evans(7) reported only one occurring in 150 cultures from this source, while more recently Kon(2) found only two in forty-seven cultures.

Whilst the majority of the coliform bacteria isolated from cream in this study appear to be of faecal type, the possibility that they have been derived more immediately from contaminated utensils cannot be overlooked.

The effect of coliform organisms upon the flavour and quality of cream.

Coliform organisms are known to produce harmful flavours and conditions in cream, and upon theoretical considerations alone their presence is undesirable. Although the relationship of the group to the production of fermented and ropy conditions is recognised, a more specific knowledge of the

consequences of the group as a whole, and more particularly the comparative effects of the *coli* and *aerogenes* forms, is lacking. It has been considered desirable, therefore, to study the effect of a number of the strains isolated upon cream flavour, noting particularly the comparative effects of different members of the group upon quality.

Experimental.

A technique of simple inoculation of fresh cream was adopted, and the normal conditions under which cream is handled during the summer were simulated as far as possible. The cultures used were grown in milk at 37° C. and subcultured for two successive days prior to inoculation. Fresh unpasteurised cream was distributed in 250 ml. lots in sterile Erlenmeyer wide-necked flasks, and each flask was inoculated with 1 per cent. of the milk culture. A control flask was similarly inoculated with 1 per cent. of sterile skim milk. The cream used was obtained a few hours after separating and had been held at 0° C. Plate counts made immediately prior to inoculation showed a count of 17,500 per ml. No gas-producing organisms were evident in a dilution of 1/100. The cream was choicest quality with a clean natural flavour and aroma. After inoculation the flasks were held at 25° C. for 24 hours. They were graded at intervals by taste and aroma with the assistance of a competent cream grader.

Results and discussion.

The extent to which the organisms studied brought about deterioration in the grade of the cream is shown in Table III.

Table III.

Group	Species	Number of strains	Number causing deterioration to second grade cream after		
			4 hours	8 hours	24 hours
Coli	*Bact. coli*	4	—	—	—
	Bact. communior	2	—	—	—
	Bact. acidi-lactici	4	—	1	3
	Bact. neapolitanus	1	—	—	—
	Bact. coscoroba	2	—	—	—
Total		13	—	1	3
Aerogenes	*Bact. aerogenes*	6	—	2	4
	Bact. cloacae	4	1	2	2
Total		10	1	4	6
Intermediate		6	1	4	5

The control flask of cream was choicest quality at the end of 24 hours with a clean mild acid flavour.

Description of flavours.

Bact. coli. The tendency of the *coli* group as evidenced by the thirteen organisms studied was to cause a general lowering of quality to first grade after about 8 hours' incubation with the production of indefinite unclean and

stale flavours and aromas. No traces of ropiness were observed. Little distinction could be made between the various species studied in their effect upon flavour and quality, only *Bact. acidi-lactici* bringing about changes sufficient to cause deterioration to second grade at the end of 24 hours. This species was possibly characterised by the production of an alcoholic aroma.

Bact. aerogenes. The *aerogenes* type cultures were characterised by a variety of fermented and gassy flavours, the diversity of effects being much wider than with the *coli* group. Ropiness was commonly produced and the condition was often associated with a rank marsh-like aroma. In marked contrast to the *coli* strains this group brought about a general lowering of quality to second grade, some species producing marked deterioration after 8 hours. Three dulcitol fermenting strains of *Bact. aerogenes* corresponding to *Aerobacter oxytocum* (Bergey(8)) each produced a characteristic animal-like flavour resembling somewhat that of colostrum. This condition is generally referred to by graders as "albuminous" or "cowy". The *Bact. cloacae* strains were sharply differentiated from *Bact. aerogenes*, in that they showed no signs of ropiness or gas formation and were distinguished by the production of sharp "chemical-like" flavours. One strain produced a very strong aroma of diacetyl.

Intermediate. The intermediate forms of the *coli-aerogenes* group showed a marked similarity to *Bact. aerogenes* in their behaviour in cream. They brought about a marked and rapid deterioration in quality accompanied generally by ropiness. Fermented and gassy conditions were frequent, and the same animal or "cowy" flavour produced by certain strains of *Bact. aerogenes* was common.

The number of organisms examined is not sufficiently large to permit of positive conclusions, but there is a suggestion that the destructive effects of the low ratio or *coli* strains in cream are not as marked as those of the *aerogenes* and intermediate forms. Generally speaking the quality of the cream was not seriously affected by the growth of the *coli* strains, even though the initial inoculations were heavier than one would expect under conditions of extreme negligence in production. With *Bact. aerogenes*, *Bact. cloacae* and the intermediate forms, a marked deterioration was frequently observed after only a few hours. The comparative results are seen more clearly in Table III, where it is seen that three (or less than 25 per cent.) of thirteen *coli* cultures caused second grade cream after 24 hours, whilst with the *aerogenes* and intermediate strains eleven out of sixteen (or 68 per cent.) lowered the quality to second grade in the same period.

Summary.

In a study of coliform organisms in cream attention has been directed towards the occurrence of specific types within the group, and to their effect upon flavour and quality.

Coliform organisms were isolated from eighty-eight (or 72 per cent.) of

112 samples of cream examined. By preliminary enrichment methods 128 strains were isolated, of which ninety-eight (or 76·5 per cent.) were typical *coli* strains, fourteen (or 11 per cent.) *aerogenes*, and sixteen (or 12·5 per cent.) intermediate.

Twenty-nine coliform organisms were tested for their effect upon cream flavour and quality. The *aerogenes* and intermediate types were found to be similar in their effect and to produce a more rapid and serious deterioration in quality than the *coli* strains of faecal type.

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102. STUDIES ON THE MYCOLOGICAL PROBLEMS OF DAIRYING

I. THE SURFACE MOULDING OF BUTTER

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(With 5 Charts.)

The most common and the most spectacular of mould defects is the surface moulding of butter. This defect seldom occurs by itself. Almost invariably it is associated with the moulding of the parchment wrapper and the wood of the containing box. In this investigation, however, the wrapping materials are considered only in so far as they may affect the development of moulds on the surface of the butter itself.

The literature contains a number of references to superficial moulds, but many of these relate to discolorations on isolated packages and bear little relation to present-day commercial conditions. It is the development of modern methods of transport and storage and the increase in the importation of butter from abroad that has brought this defect into prominence. The occasional appearance of mould has now given place to regular outbreaks of considerable commercial importance.

Except for an occasional sample, material for this investigation has been collected from butter consignments arriving at the Port of London. This has involved the study of the commercial methods in use and considerable survey work in cold stores and warehouses. The samples taken represent butter and wrapping materials from various countries, the chief of which are New Zealand, Australia, Denmark, Russia, Central Europe, South Africa and the Argentine.

Surface moulding is known to the "Trade" simply as "Mould". It refers to the blemishes of various sizes, shapes and colours which develop on the butter and the wrapping materials, affecting both the quality and the appearance of the product. It is a seasonal defect, occurring to a greater or less extent during the warmer months. The amount of butter affected cannot be usefully estimated. It is customary to settle complaints of "Mould" by means of credit notes, and it is only when these are not acceptable and defective boxes are returned that "Mould" is seen in the London warehouses. These returned boxes must represent only a small fraction of the defective butter. Spotting is confined entirely to the superficial layers, and the butter is usually scraped and repapered before being sold.

FUNGI ISOLATED.

Isolations were made from mouldy butter, parchment and boxwood. Butter and wood samples were surface sterilised before plating to avoid the isolation of chance contaminants. This was not possible in the case of parchment. From more than 2000 isolations the following fungi were obtained from definite surface discolorations on butter:

(1) *Cladosporium herbarum* (five strains).
(2) *Penicillium* spp. (nine forms).
(3) *Aspergillus* spp. (three members of the *glaucus* group).
(4) *Alternaria* sp.
(5) *Stemphylium* sp.
(6) *Fusarium* spp. (including *F. culmorum*).
(7) *Phoma* spp. (eight forms).

The first five of these were also isolated repeatedly from spots occurring on wood and parchment. *Phoma* spp. frequently cropped up in isolations, and occasionally small patches of pycnidia were observed on wood. On three occasions *P. alternariaceum*, a fungus which appeared only once from butter, developed from fragments of discoloured wood, and, in addition, *Trichoderma lignorum* was isolated several times from greenish spots on boxwood.

Certain other fungi from butter appeared from time to time. They came from no special type of discoloration and may have been present either as indistinct surface growths or as viable spores which escaped surface sterilisation. Chief of these were:

(1) *Mucor* spp.
(2) *Trichoderma lignorum.*
(3) *Verticillium* sp.
(4) *Gliocladium* sp.
(5) *Stysanus* sp.
(6) *Fusarium* spp.
(7) *Oospora* (*Oidium*) *lactis.*
(8) *Acrostalagmus* sp.
(9) *Trichothecium roseum.*

A very miscellaneous assortment of organisms also appeared in isolations from discoloured parchment. This was to be expected in view of the difficulties of surface sterilisation already mentioned. As they failed to produce typical discolorations on parchment they were considered as accidental and unimportant, so that no enumeration of them is called for.

TYPES OF DISCOLORATION.

Considered according to the frequency of their occurrence the chief discolorations found on butter may be classified as follows:

(1) A *bluish black* spot varying considerably in size and shape was most frequently encountered, and from this type *Cladosporium herbarum* was invariably isolated.

(2) A *large spreading muddy brown* blemish, showing some variation in shade, was of common occurrence, and from this several species of *Phoma* were obtained. Occasionally *Alternaria* sp. appeared from spots of this type.

(3) Definite *green surface growths* were common on badly moulded butter. *Penicillium* spp. or members of the *Aspergillus glaucus* group were isolated.

(4) *Small black spots*, sometimes liable to be confused with those due to *Cladosporium herbarum*, almost invariably gave *Stemphylium* sp.

(5) A *large orange-yellow* area appeared fairly frequently. Isolations were made from samples gathered from different sources at widely separated times. The same organism appeared on every occasion, but as yet has not been identified. It produces a well-developed aerial mycelium which is cream coloured at first but later becomes brownish with numerous golden exudation drops and with a pronounced yellowish brown colour in the medium. Up to the present, however, no spores have been produced. Thom and Shaw (6) refer to an orange-yellow discoloration caused by *Oidium lactis*, but this fungus has never developed in isolations from these discolorations.

(6) A *very large bright reddish pink* area was seen on several occasions both in New Zealand and Australian butter. The organism invariably isolated was *Fusarium culmorum*.

These discolorations were reproduced in the laboratory. Small blocks of butter were inoculated with a drop of spore suspension, dry spores or, as with No. 5 above, pieces of mycelium, and were held in a humid atmosphere. The resulting spots closely resembled those occurring naturally.

Conditions influencing amount of attack.

(1) *Moisture.*

The importance of moisture in the development of moulds on butter has been stressed by other workers, *e.g.* Thom and Shaw (6) and Macy (5), and during the course of this investigation, both in experimental work and under commercial conditions, the significance of water has been apparent.

With regard to the availability of water for fungal spores lying on the surface of butter there is first of all the water content of the butter itself. The occluded water, being surrounded by fat, is not immediately available and is of importance only after the butter is attacked. The amount and distribution of free water depends largely on the way in which the butter has been "worked" and will thus vary from sample to sample according to the method of manufacture and the amount of drying which the surface layers have undergone. No data, however, can be offered on this point.

The most important external source is the condensation water which collects on boxes of butter—wood, parchment and butter—soon after they are removed from cold store. Under humid atmospheric conditions the amount formed is considerable, and boxes dripping with condensation water have been frequently observed. The spread of mould spots by running drops of water has been apparent on many of the mouldy boxes examined. Boxes of mouldy butter returned from the retailer have been seen with the condensation water

still present. In such cases it is obvious that conditions must have been highly favourable for moulding throughout the whole period subsequent to withdrawal from cold store.

Other sources of moisture, such as rain or sea water, need only be mentioned. They have been found to be connected with outbreaks of mouldiness on several occasions.

(2) *Temperature.*

The commercial life of butter may be divided into two stages. The first and longest stage is during transport and storage, where the temperature is controlled. The second, and probably the more important stage, begins with the removal of the butter from cold store and ends only when it is consumed. The temperature during this second period is not controlled.

The growth of moulds on meat in cold store demonstrates that certain fungi at least are able to develop at low temperatures. Brooks and Hansford (2) state that several strains of *Cladosporium herbarum* are capable of growth at $-6°$ C., and Haines (1) notes that *Sporotrichum carnis* grows at $-7°$ C. He suggests that the lower limit for this fungus on dried Czapek's agar is in the neighbourhood of $-10°$ C.

The usual temperature for the transport and storage of imported butter is 15–18° F. ($-9{\cdot}4$ to $-7{\cdot}7°$ C.). Butter inoculated with spores of the fungi isolated from discolorations (p. 155) was held in cold store at the above temperature for periods varying from 3 to 12 months (some has actually been in store for more than 2 years), but no spotting whatever occurred. Microscopic examination of spores recovered from the inoculated butter revealed no signs of germination.

This result is confirmed in commercial practice. With one exception, mould growths have not, within recent years, been observed on the surface of butter when examined immediately after removal from cold store. This has been the experience of the writer and also of several inspectors of dairy produce. It would appear, therefore, that the temperature of storage and transport is sufficiently low to prevent damage by fungi. At the same time, having regard to the difficulties of maintaining desired temperatures in cold stores and ships' holds, the results of Brooks and Hansford and of Haines show that the margin of safety, $1{\cdot}7°$ C. for *Cladosporium herbarum* and $0{\cdot}7°$ C. for *Sporotrichum carnis*, is small.

After leaving cold store, butter is subjected to an endless variety of treatments. With the exception of warehouses, where temperature is controlled, the temperature of the butter is dependent on weather conditions. The rate at which discolorations appear will depend on the optimum temperature for growth of the fungus and on the season of the year. The fungi isolated from discolorations grow rapidly at normal summer temperatures and are also capable of slow growth at 1° C. The rapid development of surface defects during the summer months—for example, complaints have been registered

within 4 days of withdrawal from cold store—is no doubt due in large measure to the suitable temperature conditions prevailing.

(3) *Food available to fungi on the surface of butter.*

The amount of fungal growth and therefore the amount of spotting produced is obviously dependent on the food supplies available. Three sources appear possible, viz.:

(*a*) The butter itself.
(*b*) The wood of the containing box.
(*c*) The parchment wrapper.

(*a*) *The butter itself.*

The mere fact that butter is subject to mould attack shows that it contains suitable food for fungal growth. Macy(5) has discussed in considerable detail the various constituents in so far as they may be utilised by moulds. He finds considerable differences in the capacity of the fungi tested to make use of the different substances, butterfat, lecithin, fatty acids, curd, lactose, etc., but concludes that if conditions such as moisture, oxygen, and temperature be favourable, butter will furnish a satisfactory food supply for fungi.

Under commercial conditions some butters appear to be more liable to mouldiness than others. The point was tested with 256 samples of butter representing sixty-four brands. Thirty-two of these samples were unsalted and the remainder salted ($1\frac{1}{2}$–2 per cent. salt). The samples were inoculated on freshly cut surfaces, and the conditions of moulding, temperature, humidity and size of inoculum were controlled. After 2 weeks' incubation the samples were arranged in groups according to the size of the discolorations produced. Table I expresses the results obtained.

Table I. *The surface moulding of butter under controlled conditions.*

Group	No. of samples	Extent of moulding
1	32	×
2	18	× ×
3	28	× × ×
4	20	× × × ×
5	16 (unsalted)	× × × × ×

The results given by Table I suggest that the variation observed may be due to the varying salt content of the samples tested. While it is true that the unsalted butter developed the largest spots, the differences displayed by the first four groups, which included all the salted samples, were quite definite and continuous. The difference between groups 1 and 4, that is between two salted groups, was greater than that between groups 4 and 5, a salted and an unsalted group.

When the surface of butter becomes wet, food substances become readily available to fungal spores by passive diffusion from the butter to the surface water. Different samples of butter vary greatly in this respect. This was shown by a series of germination tests in hanging drops of water which had

been in contact with butter. The amount of water, the time in contact, and the area covered were the same in each case. The test organism was a strain of *Cladosporium herbarum*. This method was also adopted for parchment and boxwood. For the sake of comparison, therefore, the results are combined in Table V.

(*b*) *The wood of the containing box.*

The moulding of boxwood is a problem which requires special treatment. It has been studied in some detail, particularly with regard to sources of contamination, special wood fungi and the use of inhibitory substances as a means of control. All these questions, however, lie outside the scope of this paper and must be dealt with under a separate title.

The box is separated from the butter by two, sometimes three, layers of parchment. Its influence as a source of food for fungal growth in connection with surface defects of butter will thus be intimately concerned with the intervening parchment.

Free water must obviously play a large part in rendering the nutrients present in wood available for fungi which are liable to attack the butter. That such conditions arise in practice has already been mentioned.

The ability of water which has been in contact with boxwood to stimulate germination was tested experimentally. The method adopted followed the lines mentioned in connection with the similar experiment on butter. In this case the area covered was delimited by means of wax. The wax was shown by direct experiment to have no effect on the germination of the test organism.

(*c*) *The parchment wrapper.*

The importance of the parchment wrapper in connection with surface defects has long been realised. Considerable work has been done, but for the most part it has been concerned with parchment as a source of contamination and with the discussion of methods of treating parchment with hot water, brine or disinfectants with the object of producing a sterile wrapper.

The extent to which parchment may be contaminated is largely a matter outside the control of the manufacturer. The sulphuric acid bath and the subsequent drying treatment would to a large extent ensure sterilisation, and the chances of recontamination during cutting and packing are slight. The writer's experience with commercial parchment has shown that it is as a rule free from the spores of fungi which cause discolorations on butter. Contamination depends to a great extent on the methods of handling and storage and the general sanitary conditions of the dairy factory.

Under suitable conditions fungi grow on parchment, producing in some instances definite discoloured areas and in others diffuse inconspicuous growths. In both instances the surface of the butter would probably be affected, if in contact with such wrappers. It is obviously desirable to use a parchment which is not liable to support or stimulate fungal growth.

Parchments weighted with gums, sugars, etc., have been frequently used

in the past, but as they were found to be readily attacked by moulds, certain countries have specified that no weighting materials should be used. Following on this argument it has been assumed that liability to mould goes hand in hand with high water-soluble content, and manufacturers have been asked to reduce the water-soluble matter to a minimum.

Parchment is inclined to lack pliability, and to overcome this defect it has been customary to introduce a softening agent such as glycerine or salt. Danish butter is generally wrapped in a salt-softened parchment, while Australia uses a glycerine-treated wrapper. New Zealand butter is always wrapped in a hard, thick, neutral parchment.

The influence of parchment on fungal growth, therefore, raises the questions of the effect of the water-soluble content of the parchment itself and the effect of the added substances, glycerine and salt.

(1) *Effect of water-soluble matter.* In attempting to assess experimentally the liability to moulding of different samples of parchment, it was hoped by careful placing of equal amounts of inoculum to be able to express the results by actual measurements of the spots produced. This was not always possible. With parchments of high glycerine or salt content, the water which formed on the surface frequently caused the inocula to spread and thereby cause discolorations of indefinite extent. A few spores picked up on a dry point and placed on a spot which had been previously moistened was found to be the most satisfactory method of inoculation.

Estimations were made of the water-soluble content of a number of samples. The method adopted was that used by Arup(1). Table II gives as a percentage of the dry weight the amount of water-soluble matter present in eight samples.

Table II. *The water-soluble content of parchment expressed as a percentage of the dry weight.*

Sample ...	1	2	3	4	5	6	7	8
Water-soluble matter % of dry weight	0·58	0·74	0·64	1·78	0·92	1·02	1·54	1·10

The lowest figure obtained was 0·58 and the highest 1·78. These amounts agree well with those of Arup(1), who found, with one exception, a variation of 0·55–1·65.

To estimate the value of the water-soluble matter as a source of carbon for fungal growth, various concentrations of an extract from parchment were added to a basal medium composed of the mineral salts of Dox's solution. A parallel series with glucose as the sole source of carbon was carried on at the same time. The results for a strain of *Cladosporium herbarum* are shown in Chart 1, where the dry weights of the mycelia produced are plotted against the logarithms of the concentrations of carbon.

Both from the appearance of the actual cultures and from the dry weights as shown above, it is evident that the water-soluble matter is a useful source

of carbon food. It may be said that under the conditions of experiment, the water-soluble extract possesses one-third the efficiency of glucose as a carbon source.

Weak solutions of the water-soluble matter definitely stimulate the germination of spores. Spores of two strains of *Cladosporium herbarum* and a species of *Penicillium* were germinated in a series of dilutions of a concentrated extract, 7·4 per cent. solids, of parchment. Germ tubes were measured after 24 hours at 20° C. In Chart 2, the total length of the germ tubes of 100 spores is plotted against the concentration of the extract.

The reduced germination shown in the figures at the higher concentrations was only a temporary effect. After a further 24 hours, mycelial wefts with

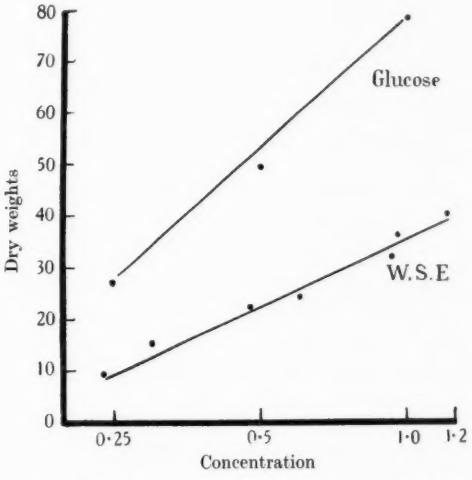

Chart. 1. Illustrating the comparative value of glucose and the water-soluble extract of parchment as sources of carbon.

crops of spores arising had developed in the strongest extracts and the maximum of growth was now at that end of the series. In the weakest extract and in distilled water, growth had come to a standstill. The germ tubes in the latter were thin and obviously starved, whereas in the stronger solutions they were stout and vigorous.

These experiments show that the water-soluble matter of parchment definitely stimulates the germination of fungal spores and is readily utilised by moulds as a source of carbon.

How far the liability to moulding of different parchments runs parallel with their water-soluble contents is illustrated in Table III. A series of parchments with increasing water-soluble content was prepared by first removing as far as possible the water-soluble matter present and then soaking in different concentrations of extract for 40 min. These papers, together with three

samples of commercial parchment, were inoculated with two strains of *Cladosporium herbarum* and placed in a saturated atmosphere at laboratory temperature.

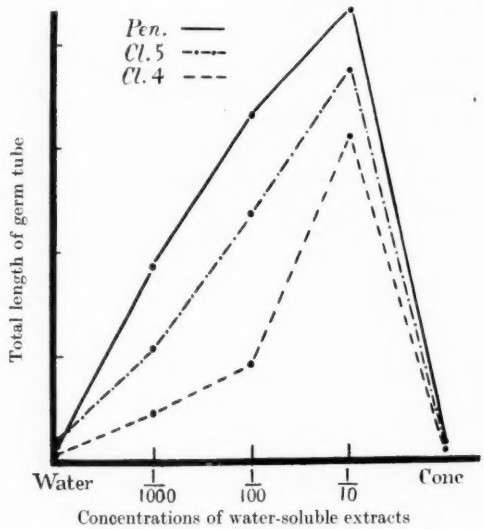

Chart 2. Showing the germination of spores in different concentrations of water-soluble extract.

Table III. *Moulding in relation to the water-soluble content of parchment.*

No.	Description of parchment	Water-soluble content	Strain 4	Strain 2
1	Washed	0	×	×
2	Commercial	0·74	×	×
3	,,	0·92	×	×
4	,,	1·54	×	×
5	Soaked series	0·90	×	×
6	,,	1·90	× ×	× ×
7	,,	3·90	× ×	× ×
8	,,	7·80	× × ×	× × ×

The discolorations formed in the higher members of the series were larger and darker in colour than those produced on the lower members. It was, however, impossible to make any real distinction between the three commercial papers. In some instances the growth, when examined under the microscope, did appear to be more luxuriant on parchment No. 4 than on parchment No. 2, but this was not always evident and to the naked eye was not noticeable. The fungi tested were able to penetrate the parchment with ease. It is important to note that the washed parchment produced a spot only slightly weaker in colour than those formed on the commercial papers.

While the data given in Table III show that there is some relation between the water-soluble content of parchment and the intensity of moulding, it will

be observed that the reduction of the water-soluble content to a minimum does not eliminate moulding. This may be due to the technical difficulties of removing all the water-soluble matter, even with repeated washing, or to the capacity of the fungus to attack the insoluble material of the parchment. From the practical point of view, it would seem that to strive after a water-soluble content below that at present existing in good quality parchment is not likely to give results of any commercial value.

In the experiments to which Table III refers, the parchment was kept in a humid atmosphere. A further series was carried out in which the papers were moistened with distilled water before inoculation. Growth was more rapid in this series and much larger discolorations were produced. The difference between the spots on papers of high water-soluble content and those of low, remained clear; but again it was practically impossible to distinguish between the commercial parchments. As before, the washed parchment developed appreciable discolorations.

Parchment, however, cannot always be considered alone. In practice well-wrapped butter has two or three layers of parchment. It is important to consider the effect of the water-soluble matter when the paper is in contact with butter, and also to ascertain whether contact with wood influences the capacity of the parchment to support fungal growth.

Testing again with a strain of *Cladosporium herbarum*, a prepared series and one sample of commercial paper were placed on butter, inoculated and held for 14 days in a saturated atmosphere at 20° C. Spotting developed rapidly and measurable discolorations appeared in less than a week. Table IV gives the diameters of the spots and indicates the intensity of the colour produced.

Table IV. *Parchment on butter. Showing the size of discolorations and intensity of colour in relation to the water-soluble content.*

Water-soluble content	Average diameter	Colour
7·8	5·5	× × × ×
3·9	5·2	× × ×
1·95	5·0	× × ×
0·97	4·7	× ×
0·70	3·5	×
0·00	3·5	×

Little difference was to be seen in the size of the spots although the colour was more pronounced in the higher members of the series. Contact with butter definitely masked the effect of the water-soluble content.

A similar series was placed in cold store at −9° C. for 24 hours and then at 20° C. for 14 days. Condensation water collected on the surface of all papers. Owing to the spread of the inoculum it was impossible to measure satisfactorily the resulting discolorations. It was apparent that the free water enabled the fungus to develop more rapidly and to produce larger spots. The distinction between the spots produced was even less noticeable than in the

previous experiment. The spots on the paper from which the water-soluble matter had been removed developed less colour than any of the others.

It has already been mentioned that, when water is in contact with butter or boxwood, substances that definitely stimulate germination diffuse into the water drops. A similar experiment was carried out with parchment. The samples of parchment tested consisted of a washed sample, a commercial paper and a prepared series, the highest member of which contained 7·4 per cent. of water-soluble matter. They appear in this order in Table V. In order to assess the comparative value of the butter, the boxwood and the water-soluble matter of the parchment, the results are collected in Table V.

Table V. *Germination of* Cladosporium herbarum *in water after contact with parchment, boxwood and butter. The figures represent the total length of germ tubes of* 100 *spores.* (*Micrometer divisions.*)

No. of sample	Parchment	Wood	Butter
1	110	60	56
2	180	324	180
3	198	392	408
4	254	444	540
5	262	700	772
6	330	1370	820

It will be observed that with the exception of two samples of wood and two of butter, the stimulus given to germination even by parchment of high water-soluble content is exceeded by all the samples of wood and butter.

(2) *Effect of addition of glycerine.* A number of experiments similar to those in the preceding section were carried out with glycerine treated paper. Glycerine itself was shown to have a definite food value for the common moulds isolated from parchment, but weight for weight it was much inferior to glucose. In weak solutions glycerine stimulated germination, and both the percentage germination and the average length of germ tube increased with the concentration up to 1 per cent. Higher concentrations were not tested.

A series of parchments with increasing amounts of glycerine were prepared for experiment. Under controlled conditions the extent of the moulding followed the glycerine content. The hygroscopic nature of the higher members of the series permitted the rapid spread of the discolorations and the formation of numerous subsidiary spots.

When in contact with butter, glycerine-treated papers showed discolorations sooner and to a greater extent than glycerine-free ones. Chart 3 plots the diameters (average of ten spots) after 7 and 13 days.

The interrelation of temperature, glycerine content of parchment and amount of moulding is brought out in Chart 4.

The crossing of the two lower curves is a point of practical interest. With glycerine-free papers there is no spotting at 1° C. after 6 weeks, whereas at 10° C. it develops fairly rapidly. With papers of high glycerine content, there is pronounced spotting at 1° C. after 6 weeks. The beneficial effect is thus relatively much greater with glycerine-free than with glycerine-soaked papers.

In other words, the addition of glycerine, even as little as 1 per cent., to a large extent annuls the value of low temperatures in repressing discolorations.

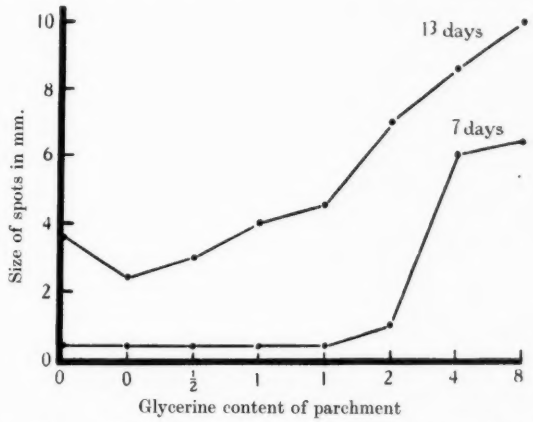

Chart 3. The moulding of glycerine-softened parchment in contact with butter after 7 and 13 days at laboratory temperature.

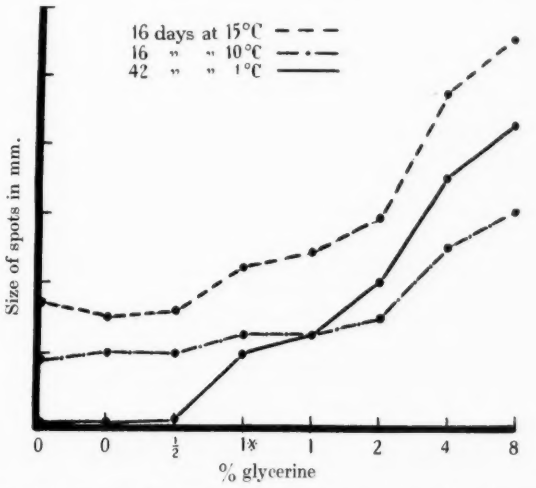

Chart 4. Illustrating the moulding of glycerine-softened parchment at 1, 10 and 15° C.

It is clear from these experiments that glycerine has a definite food value and that it stimulates germination. In addition its hygroscopic nature increases the water-holding capacity of the parchment to which it is added. Papers containing much glycerine not only mould to a greater extent, but they develop visible spots more quickly and the spread of infection is increased.

At low temperatures spots are able to develop on glycerine-softened parchments while untreated parchments remain clean.

(3) *Effect of addition of salt.* Treatment with salt during manufacture produces a pliable parchment and one which is said to resist attack by fungi. The retarding effect of salt on fungal growth has long been realised by the dairy industry, and the sterilisation of parchment with hot brine before wrapping has been recommended by several workers and is a practice adopted by many factories. The aim of this treatment is the production of a sterile, mould-resisting parchment.

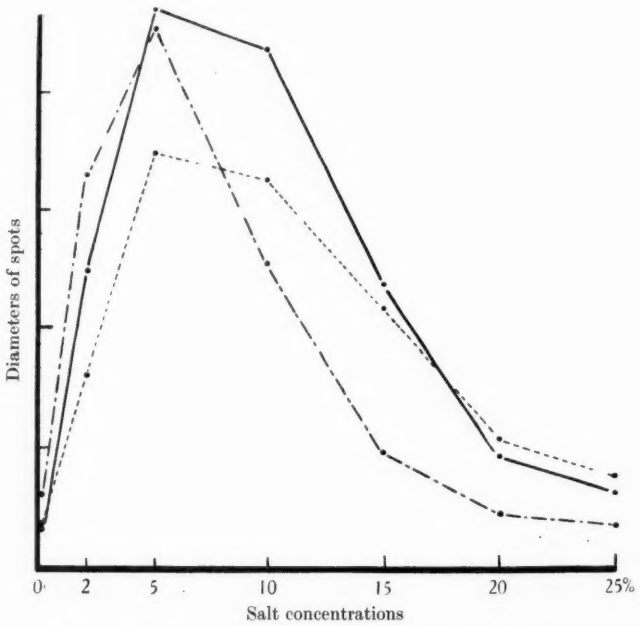

Chart 5. Illustrating the effect of salt on the spotting of parchment in contact with butter.

The capacity of salt-treated parchment to resist fungal attack appears to rest on little experimental basis. The salt tolerance of fungi has been investigated by many workers, and the results show that the concentration of salt required to inhibit fungal growth varies considerably with different fungi. Grimes, Kennelly and Cummins (3), for example, using Czapek's solution plus salt on a number of fungi isolated from butter, found that while 10 per cent. salt inhibited growth in some cases, many fungi grow slightly in 15 per cent. and a few required 20 per cent. salt to prevent growth.

How far the addition of salt prevents the spotting of parchment is illustrated in Chart 5. The testing was carried out with two strains of *Cladosporium*

herbarum and a species of *Penicillium* on a series of salt treated parchments in contact with butter. Discolorations developed first on parchments soaked in 5 and 10 per cent. salt, and later these papers showed the largest spots. The least attack was found on the salt-free parchment.

The effect of adding a moderate amount of salt is to increase the intensity of spotting, while the use of still higher concentrations diminishes it. Nevertheless, the highest concentration used, 25 per cent., did not produce a more favourable result than untreated parchment. It is true that salt has no value as a nutrient and that at high concentrations it represses growth by its osmotic action, but any favourable effect so arising is more than counterbalanced by its tendency to cause accumulation of water. On the basis of the above experiment the practice of adding salt to parchment is not to be recommended.

SUMMARY.

1. Six types of surface discolorations are described and a list is given of the fungi causing these discolorations. The fungi listed are shown to be capable of reproducing the discolorations under experimental conditions.

2. The surface moulding of butter is shown to depend largely on the weather conditions at the time of removal from cold store (temperature and humidity), and the foodstuffs available from the wrapping materials.

3. The butter, boxwood and parchment are capable, in the presence of free water, of supplying substances which stimulate the germination of fungal spores.

4. The water-soluble extract of parchment stimulates fungal germination and growth. Its value as a nutrient is masked when parchment is in contact with butter. Even a well-washed parchment is capable of supporting fungal growth.

5. The butter and the boxwood are even more important as sources of soluble food material than parchment of high "water-soluble" content.

6. Glycerine is a source of food for fungi and a glycerine-softened parchment is more liable to mould attack than a glycerine-free paper.

7. It has been shown that the softening of parchment with salt does not prevent mould attack. Two strains of *Cladosporium herbarum* and *Penicillium* sp. attacked salt-softened parchment more easily than untreated parchments.

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637.225:582.28

103. STUDIES ON THE MYCOLOGICAL PROBLEMS OF DAIRYING

II. THE INTERNAL AND SUBSURFACE DISCOLORATIONS OF BUTTER

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(With 1 Plate and 1 Chart.)

Most of the butter imported by Great Britain is salted and as a general rule contains approximately 1½–2 per cent. of salt by weight. Small quantities, often made under contract for special purposes, may contain more or less salt as the case may be, and there is always a steady demand for unsalted or fresh butter.

Internal discolorations occur in unsalted and lightly salted butters. Under commercial conditions they have not been observed in normally salted butter. The appearance of an affected box depends largely on the degree of infection and the length of time the box has been out of cold store. Pl. I, fig. 1, illustrates a defective box which has been out of store for about 3 weeks. In this case all the discolorations which can be seen from the outside lie actually below the surface, and the appearance is quite distinct from surface blemishes. Normal surface growths sometimes, but by no means invariably, accompany internal spotting.

The most common type of subsurface discoloration is bluish black in colour. Considerable variation is observed in the size and shape of the spots, but as a general rule they are more or less circular and seem to reach their maximum size at a diameter of between 1 and 2 cm. Other types of subsurface discolorations were observed, the most noticeable being a rapidly spreading one of a muddy brown colour. The organisms found in association with this type were species of *Phoma*.

All the internal, *i.e.* deeply seated, discolorations were of the blue-black type. Muddy brown areas were not found more than 2 cm. below the surface.

The depth to which these discolorations occur may be gathered from Pl. I, fig. 2. This shows a section cut through the centre of a 56-lb. block. The spots at the centre are at least 14 cm. from any surface.

Methods of isolation.

Isolations were made on plain and acid malt agar in the usual way, but on account of the low oxygen conditions that must exist inside a large block of butter, isolations were also made under reduced oxygen conditions. Freshly

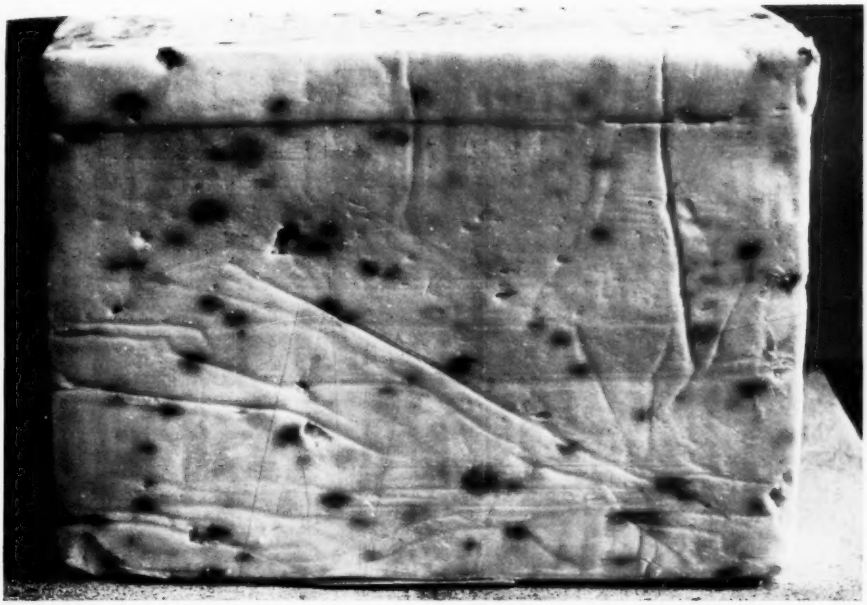

Fig. 1.

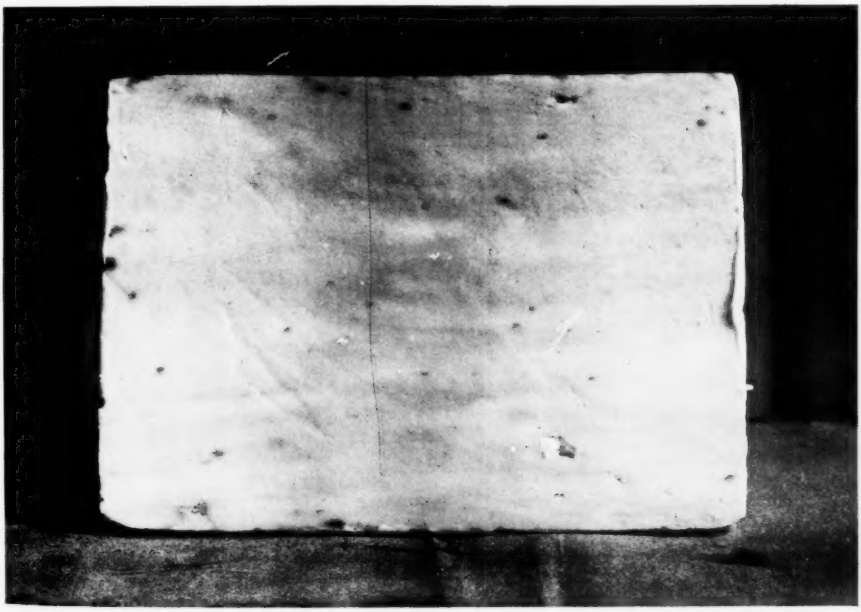

Fig. 2.

poured medium was used and the plates were placed in small desiccators over pyrogallol or incubated in an atmosphere of commercial nitrogen.

ORGANISMS.

A large number of isolations were made from blue-black internal and subsurface discolorations. Strains of *Cladosporium herbarum* were almost invariably isolated. In culture these strains showed almost continuous variation from a very dark almost black heavily sporing type to a light-coloured strongly mycelial type. From this variety of cultures six strains were selected for further experiment. The strains chosen may be regarded as representing the range of variation encountered and as including the most frequently occurring types. Table I shows the frequency of occurrence of the various strains in isolations from four large samples.

Table I. *The occurrence of strains of* Cladosporium herbarum *in isolations from internal and subsurface discolorations.*

	Strains of *C. herbarum*					
Sample	1	2	3	4	5	6
1	4	8	27	25	34	0
2	0	16	20	16	10	0
3	6	4	16	21	9	3
4	8	18	10	42	19	1
Totals	18	46	73	104	72	4

All these strains except the last had appeared frequently in isolations made from surface discolorations.

The cultural characteristics of the above strains were definite and distinct. The method of spore formation was the same for each strain and was typical of *Cladosporium herbarum*. The tendency of the first formed conidia to bud and form masses of small almost spherical conidia (the *Hormdendron* type) varied from strain to strain. Most of the conidia formed by strains 1, 2 and 4 were of this type, and large spores of the *Cladosporium herbarum* type were rare. In strain 5, large dark one-septate spores were common, and strain 3 also produced them in considerable numbers. Table II gives the spore size for the various strains (average of 100 spores in each case), the colour and the amount of aerial mycelium after 6 weeks' growth on Dox's agar at 20° C.

Table II. *Strains of* Cladosporium herbarum *after* 6 *weeks' growth at* 20° C. *on Dox's agar.*

Strain	Colour	Aerial mycelium	Spore size in μ
1	Cream	× × × ×	6·7 × 3·1
2	Bluish green	× × ×	6·4 × 2·7
3	Olive green	× ×	9·2 × 4·6
4	Very dark olive	×	5·6 × 3·5
5	Olive green	× ×	13·0 × 5·0
6	Cream	× × × ×	14·6 × 5·4

Reproduction of defect.

Two methods were employed for this purpose. In the first place small cubes of imported unsalted butter were cut in two and inoculated either with spore suspension or dry spores. The pieces were then pressed together and the join sealed by heat. Incubation was carried out at room temperature in a humid atmosphere. All six strains of *Cladosporium herbarum* were tested in this manner. Apart from a tendency for the fungus to develop more strongly along the line of join and with the exception of strain 6 which did not produce colour until after 3 weeks, the rate at which the spots developed and the type of spot produced were approximately the same as in naturally infected butter.

Internal contamination obviously takes place during manufacture. In order to approximate more closely to natural conditions of infection, a sample of butter was specially prepared from sterile cream and inoculated during the "working". Four strains of *C. herbarum* were used. Two ml. of spore suspension formed the inoculum for each 5 lb. of butter. The inoculated butter was made up into ½-lb. pats and wrapped in sterile parchment. Before inoculation 1 lb. of each churning was put aside as a control. The butter was held at laboratory temperature (20–23° C.). Table III shows the extent of the spotting and the time taken.

Table III. *Showing the rate of internal spotting at* 20–23° C.

	Time in days			
Strain	4	5	6	10
1	—	—	×	× × ×
2	Trace	×	× ×	× × × ×
4	—	—	Trace	× × ×
5	—	—	Trace	× × ×

The spots produced were identical in appearance with those occurring naturally. The minimum time of 4 days and the period of 6 days required for all strains agree closely with practical experience.

Complaints of the occurrence of internal moulding have been received within a week of the removal of the butter from cold store. On one occasion twenty boxes of an infected shipment were examined immediately after withdrawal from cold store. All the boxes were found to be clean. They were then placed in a warehouse and examined from day to day. After 5 days several tiny spots were noticed just below the surface of five out of the twenty boxes. In a week, ten boxes showed definite spots. The average temperature during this time would be about 20° C.

CONDITIONS INFLUENCING THE DEVELOPMENT OF INTERNAL SPOTS.

(1) *Temperature.*

There are three stages in the history of infected butter during which temperature may exert a definite effect on the development of internal discolorations:

(*a*) The period between infection and cold storage.
(*b*) The period in cold storage.
(*c*) The period after cold storage.

(*a*) After infection there is a variable period, depending largely on factory practice, until the butter reaches cold-store temperatures. During that time

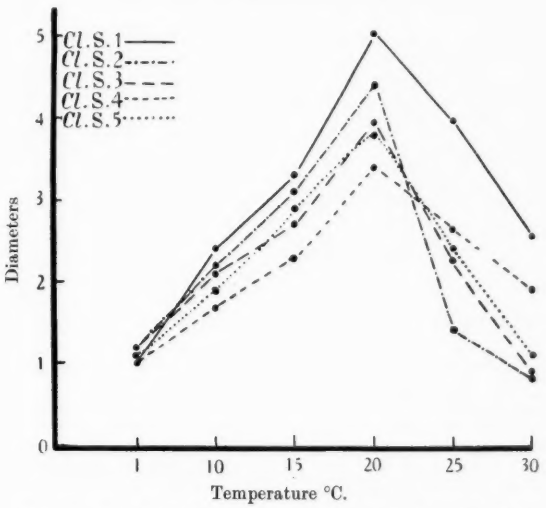

Chart 1. The effect of temperature on the rate of growth of five strains of *Cladosporium herbarum*.

the butter may be at a temperature that would permit fungal growth, and it is possible that internal spots may be present before the butter reaches cold store. In any case the growth of the organisms during this period, even if actual spots are not produced, is to be avoided. The strains of *C. herbarum* are capable of growth over a wide temperature range and grow most rapidly at normal summer temperatures. Chart 1 shows the effect of temperature on the rate of growth of five of the strains under consideration.

(*b*) The need for definite knowledge of the relation of internal moulding to temperature was emphasised during an outbreak when approximately 10 per cent. of an infected consignment had subsurface spots when withdrawn from cold store. In consequence, certain experiments were carried out not only to ascertain whether the present cold-store temperatures were sufficiently

low, but also to get data on the capacity of the various strains of *C. herbarum* to grow at temperatures approaching those of cold storage.

Cubes of butter were inoculated with the six strains in the manner previously described. Incubation was carried out at the following temperatures 28–32, 24–28, 20–24, 16–20 and 15–18° F., the average temperature being nearer the lower figure mentioned. The cubes were also inoculated on the surface, and the development of the fungus from this inoculation served to indicate when it was advisable to cut open a cube for examination.

Table IV shows the extent of spotting produced by the several strains after $3\frac{1}{2}$ months' storage at the temperatures stated.

Table IV. *The internal spotting of butter at low temperatures by strains of* Cladosporium herbarum.

Temp. °F.	Strain 1	2	3	4	5	6
28–32	×	×	× ×	× × ×	× ×	×
24–28	0	×	× ×	× ×	× ×	×
20–24	0	×	×	×	×	0
16–20	0	0	0	0	0	0
15–18	0	0	0	0	0	0

A further experiment was carried out at these temperatures using a nutrient medium in place of butter. In order to avoid the formation of ice, the medium, 2 per cent. malt agar, was dried. After inoculation the plates were kept in a closed vessel over ice. Table V shows the number of days required for germination at the various temperatures.

Table V. *Time in days for germination of* Cladosporium herbarum *on dried malt agar at low temperatures.*

Temp. °F.	Strain 1	2	3	4	5	6
28–32	30	30	8	30	13	6
24–28	56	56	35	56	24	20
20–24	—	—	—	—	82	70
16–20	—	—	—	—	—	—
15–18	—	—	—	—	—	—

In addition, experimental unsalted and lightly salted butter made from sterile cream and inoculated during the "working" was stored for 6 months under commercial conditions. No spots whatever developed during storage, but after withdrawal typical discolorations developed within a week.

It would appear from these experiments that the fungi causing internal discolorations are incapable of developing at cold-store temperatures. The lowest temperature at which growth was obtained was 20–24° F. (−6·7 to −4·4° C.), and at this temperature only two of the six strains tested showed any growth after nearly 3 months storage on dried malt agar. Four strains, however, produced small spots in butter at this temperature in $3\frac{1}{2}$ months.

(*c*) The rate at which spots may develop after delivery from cold store is a matter of great importance to the retailer. The rate of spotting is largely

a question of temperature. Unsalted butter inoculated with five strains of *C. herbarum* developed spots in 7–9 days when stored at 59–62° F., in 12–14 days at 50–54° F. and 21–24 days at 35–40° F. This explains the absence of internal spotting during the winter months.

(2) *Salt.*

Salt was added to butter made from sterile cream so as to give a series containing approximately 5, 10 and 15 per cent. of salt in the butter serum. One-quarter of the butter was not salted. Inoculation was again carried out during the "working". Table VI shows the time taken for internal spots to develop.

Table VI. *Effect of salt on the rate of internal spotting.*

		Time in days								
Strain	Salt %	4	5	6	10	12	16	20	25	30
1	0	—	—	×	× × ×	—	—	—	—	—
2		Trace	×	× ×	× × × ×	—	—	—	—	—
4		—	—	Trace	× × ×	—	—	—	—	—
5		—	—	Trace	× × ×	—	—	—	—	—
1	5	—	—	—	× ×	× × ×	—	—	—	—
2		—	—	—	× ×	× × ×	—	—	—	—
4		—	—	—	× ×	× × ×	—	—	—	—
5		—	—	—	× ×	× × ×	—	—	—	—
1	10	—	—	—	—	—	× ×	× × ×	—	—
2		—	—	—	—	—	× ×	× × ×	—	—
4		—	—	—	—	—	× ×	× × × ×	—	—
5		—	—	—	—	—	Trace	×	—	—
1	15	—	—	—	—	—	—	—	×	× ×
2		—	—	—	—	—	—	—	×	×
4		—	—	—	—	—	—	—	× ×	× × ×
5		—	—	—	—	—	—	—	×	×

It will be seen that salt retards the development of internal spotting considerably and that there are slight differences between strains. Whereas the unsalted butter showed spots within 6 days, when the salt concentration of the serum was 15 per cent. the spots did not develop until nearly 25 days after inoculation. It should be noted that this experiment was carried out during the summer when the temperature was favourable to the growth of these moulds.

A normally salted butter contains from 10 to 15 per cent. of salt in the butter serum, so that internal spotting would not be expected to appear until nearly 3 weeks after its removal from cold store. As a general rule it would be consumed within this time. It is probably for this reason that internal spotting is in practice only reported from unsalted or lightly salted butter. The experiment indicates that it would develop in all types if a sufficient time were allowed.

Although the development of internal spotting may be delayed by the addition of salt, the treatment of infected butter with salt involves difficulties which tend to eliminate this measure as a means of control. Over and above the technical difficulties or inconveniences involved, there is the fact that

such butter after treatment would no longer conform with its original trademarks and would be open to suspicion on that account.

The present work shows that, as consumption within a limited period cannot be guaranteed, a dealer can do nothing to prevent deterioration. Control of this defect obviously lies in the dairy factory. This was a phase of the problem that could not be investigated by the writer. It can only be suggested that in view of the rapidity with which this defect develops in unsalted butter, factories making butter of this type must take every precaution to avoid contamination with the spores of *Cladosporium herbarum*.

SUMMARY.

1. A description is given of internal and subsurface discolorations in unsalted and lightly salted butter.

2. Six strains of the causative organism *Cladosporium herbarum* are described.

3. These strains are shown to be capable of reproducing the defect under experimental conditions.

4. Four of the strains tested proved capable of slight growth at 20–24° F. (−6·6 to −4·4° C.) after 3½ months.

5. None of the strains grew at normal cold store temperatures, 15–18° F. (−9·4 to −7·7° C.).

6. Salt retards the development of internal spotting. Under favourable conditions it was shown that discolorations developed in 6, 10, 16 and 25 days in butter containing 0, 5, 10 and 15 per cent. of salt in the butter serum.

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104. STUDIES IN CHEDDAR CHEESE

IV. OBSERVATIONS ON THE LACTIC ACID FLORA OF CHEDDAR CHEESE MADE FROM CLEAN MILK

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(With 1 Plate and 3 Figures.)

Although a number of investigations of the flora of commercial Cheddar cheese have been published, no systematic study of the bacteria in such cheese made from milk of very low bacterial count and in the light of modern bacterial taxonomy appears to have been made. In this paper are reported the experimental findings on the nature of the true lactic acid bacteria occurring in Cheddar cheese made from Shorthorn milk of certified standard (plate count under 30,000 (actually about 1000) and *coli* absent in 1 ml.) at different stages of the ripening, and to discuss the factors controlling their frequency distribution.

Method of isolation.

Borings of cheese were taken, the outer portion to the extent of $\frac{1}{2}$ in. removed, placed in sterile Petri dishes and small portions dissected out under aseptic conditions and emulsified in saline at 45° C. Dilutions were then plated in dextrose agar, yeast extract dextrose agar, orange extract dextrose agar and litmus lactose agar. A second layer of medium was added when the first layer had cooled, to ensure the microaerophilic conditions suitable for the types of bacteria sought. The plates were then incubated at 28° C. for from seven to ten days, when well-isolated colonies were transplanted into litmus milk (using a low power microscope) and incubated at 28° C. When growth had taken place, as shown by acid production, the culture was examined, and, if satisfying the conditions mentioned below, purified by streaking and replating. The colonies were invariably white or colourless and lenticular in shape, but varied greatly in size. Selection was made to correspond as far as possible with the proportion in which they occurred. Strains are identified by numbers such as 1·4, indicating the 4th strain isolated from the 1st series (see Table IV).

Definition of the group.

Following the classification of Orla Jensen (1) the true lactic acid bacteria may be defined as having the following properties:

(1) They are gram-positive, non-motile cells without endospores.

(2) The cells obtain their energy by fermentation of carbohydrates and higher alcohols, mainly to lactic acid.

(3) Complex nitrogenous substances are required for growth.

(4) Gelatine is not liquefied and exogenous proteolytic enzymes are not produced (*Str. liquefaciens* excepted).

(5) Catalase is absent and nitrates are not reduced. Surface growth on agar media under aerobic conditions is poor.

In the present investigation 248 colonies were transplanted and cultured from eighteen cheese at different stages of ripening, from the curd at "Cheddaring" to eighteen months old cheese. Of these 247 proved to be true lactic acid bacteria according to the above definition. It is evident, therefore, that the bacteriology of clean milk Cheddar cheese is concerned almost entirely with lactic acid bacteria, and that the ripening processes must be due to the enzymes of these bacteria together with those of rennet and the original enzymes of the milk, if any. Of the 247 purified strains 117 were streptococci and 130 lactobacilli. Using Orla Jensen's classification all the cocci were found to be included in the genus *Streptococcus* and all the rod forms in the genus *Streptobacterium*. The former grew rapidly in milk, and no strains having any of the characteristics of the Betacocci (1) were obtained. The rod forms all fermented mannitol, dextrin, salicin and aesculin, and mostly grew fairly fast in milk. The Betabacteria do not ferment these sugars and grow but poorly in milk. Certain strains which resembled the Betabacteria in growing very slowly in milk were subsequently shown to be weakened forms of *Sbm. plantarum*. Further, gas was not formed in milk, and no strains gave the distinctive, dryish, raised colonies on the surface of dextrose agar slopes that are so characteristic of the Betabacteria. Further, all the lactobacilli grew fairly well at 15° C., but failed to do so at 50° C.; they fermented sucrose, maltose, salicin, dextrin and arabinose. The Thermobacteria do not grow at 15° C., grow well at 50° C. and do not ferment the sugars mentioned. Moreover, the final acidities produced in milk (1·0–1·5 per cent. lactic acid) were higher than those produced by the Betabacteria and lower than those produced by the Thermobacteria. The genera *Thermobacterium*, *Betabacterium* and *Betacoccus* are thus apparently not represented to any significant extent in English Cheddar cheese made from clean milk. The media used have been shown to give good growth with type cultures of Thermobacteria, Betabacteria and Betacocci. The absence of these types in the cheese is therefore not an apparent absence due to inability to grow in the media used for purposes of isolation. The types that do occur, however, constitute a perfectly homogeneous group physiologically, the streptococci differing from the lactobacilli mainly in their comparative rates of growth in milk. All the strains isolated grew almost as fast at 22° C. as at 28 or 37° C. and quite well at 15° C. Growth did not take place at 50° C. (Fig. 1). (A slight acidity in litmus milk was obtained with some strains of *Str. lactis* at 50° C., indicating a possible relationship with *Str. faecalis*.) The cultural characteristics of the representative types are given

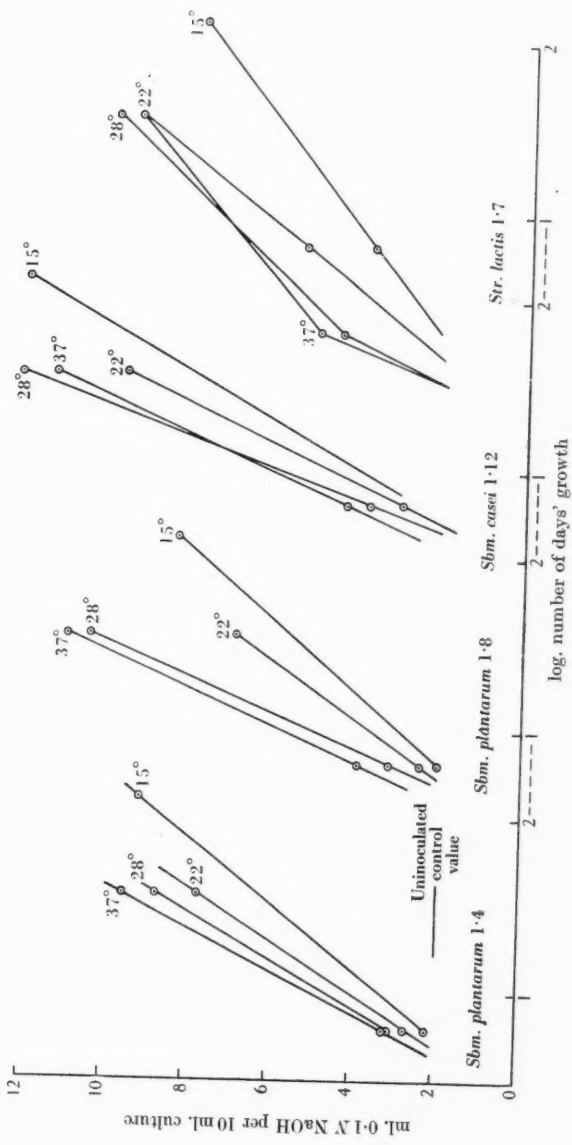


Fig. 1. The effect of temperature on the rates of acid production by lactic acid bacteria in milk.

in Tables I, II and III. When consideration is given to all the characteristics investigated, the strains fall into four well-defined groups, of which the prototypes are: (1) *Str. lactis* (Lister), (2) *Str. cremoris* (Orla Jensen), (3) *Sbm. plantarum* (Orla Jensen); and (4) *Sbm. casei* (Orla Jensen). The Danish investigator gives other species of streptococci, but in view of the fact that their separate identities as species are open to suspicion, and that none of the strains studied appeared to differ from the two types mentioned sufficiently to justify the use of other species, only these two have been recognised. This conclusion is supported by the findings of Hucker (2), who recognises four species on the basis of agglutination and agglutinin absorption studies, viz. (1) *Str. lactis* and *Str. faecalis*, (2) *Str. cremoris*, (3) *Str. thermophilus* and (4) *Str. liquefaciens*, also related to *Str. glycerinaceous*. The latter two were not obtained from the cheese examined.

The two species of lactobacilli were morphologically well defined and differed markedly in their manner of growth in milk. Their sugar fermentations were, however, identical, suggesting a different ability in adaptation to milk as a medium, by stable variants of a common progenitor. The essential differences between the species accepted may be given as follows:

Streptococci. Str. lactis forms egg-shaped cells occurring chiefly in pairs but occasionally in short chains. It gives a heavy turbid growth in dextrose broth in 24 hours at 28° C., and forms acid in arabinose, mannitol, maltose and dextrin.

Str. cremoris forms short or long chains of round or octagonal cells. Sometimes the cells are kidney-shaped, when the chain consists of diplococci. Turbidity is never observed in the dextrose broth that gives a turbid growth of *Str. lactis*, and only after 48 hours at 28° C. is a visible growth obtained as a slight sediment. Arabinose, mannitol, maltose and dextrin are not fermented.

The rate and type of growth in litmus milk, final acidity, etc., and colony form were found to be useless for identification purposes, as these properties are a function of the vigour of the particular strain in question.

Lactobacilli. Sbm. plantarum occurs in milk as rather short (2–4μ) rounded rods occurring chiefly in pairs and small clumps. Growth in milk is very slow, and a month is usually required to clot the medium, while the litmus is never reduced. A final acidity of 1·0–1·1 per cent. lactic acid is obtained at 28° C. Cultures in flasks of whey give a very turbid growth.

Sbm. casei in milk cultures forms long, slender, straight-ended rods in long chains, frequently granulated, and resembling the classical "Bulgaricus" type. It grows rapidly in milk at 28° C., clotting in from 10 to 14 days, and reduces the litmus at the bottom of the tube. A final acidity of about 1·5 per cent. lactic acid is produced at this temperature. Turbidity is not observed in flasks of whey inoculated with *Sbm. casei*.

Sugar fermentations are useless for the purpose of differentiation. It is interesting to note that cultures in dextrose broth could not be distinguished one from the other. Under aerobic conditions surface colonies of

Table I. *Cultural characteristics of lactic acid bacteria isolated from Cheddar cheese.*

Type	Morphology (in litmus milk)		Colony form and size (dextrose agar)		Growth in dextrose broth after 48 hours at 28° C.	Average time in days required to produce changes* in litmus milk at 28° C. (single colony inoculated)					Average final acidity in separated milk (% lactic acid)	Final *p*H in litmus milk
	Cell shape	Arrangement of cells	Surface	Depth		A?	A	A+C	RB	R		
Streptococci:	At time of clotting											
Cremoris	Octagonal, round or kidney-shaped, often pleomorphic, size variable	In chains of varying lengths	Very small	Small	Liquid clear—slight sediment	2	—	3	—	1–2 (before A+)	0·8	4·5–4·8
Intermediate	Round or oval	In pairs and short chains	Small	Small	Liquid slightly turbid—sediment	—	2	3	—	1–2	0·8	4·5–4·8
Lactis	Oval	In pairs, occasionally short chains	Medium or large	Medium or large	Liquid very turbid—heavy sediment	—	2	3	—	1–2	0·8	4·5–4·8
Lactobacilli:	At 0·7 % lactic acid				In whey flasks							
Plantarum	Short, rounded ends	In pairs, cells frequently curved	Small transparent	Small	Turbid—little or no sediment	10	14 or more	—	—	∞	1·0	4·0
Intermediate	Medium sized, pointed ends	Singly, pairs and short chains	Large flat	Small	Slightly turbid—sediment	8	13	21	21 (erratic)	Anomalous	1·3	3·9
Casei	Long, thin, square-ended rods, frequently granulated	In long chains, frequently thread-like	Small to medium	Small	Clear—heavy sediment	6	8	12	8	15	1·5	3·8

The rate of growth of lactobacilli in litmus milk decreased with increasing age of the cheese from which they were isolated.

*A? = mauve-pink (=0·55 % lactic acid).
A = pink (=0·70 % lactic acid).
A+ = bright pink (=0·90 % lactic acid).
RB = reduction of litmus at bottom of tube.
R = reduction of litmus.
C = clot.

Sbm. plantarum tended to be "watery", and those of *Sbm. casei* white and, when young, "curdy" in appearance.

Intermediate types. A number of strains were found to share properties of both species, i.e. some streptococci were morphologically intermediate between

Table II. *The behaviour of streptococci in dextrose broth after 48 hours' growth.*

Series	Morphology	Clear	Doubtful	Turbid
5	*Lactis*	0	0	5
6	,,	0	0	5
7	,,	0	0	3
9	,,	0	0	3
10	,,	0	0	6
5	Intermediate	0	0	2
7	,,	2	1	2
8	,,	0	0	3
10	,,	1	0	0
8	*Cremoris*	2	0	0
9	,,	4	0	0
10	,,	6	0	0

Coefficient of correlation between turbidity in dextrose broth and nature of species = 1.

Table III. *Sugar fermentations of various lactic acid bacteria.*

Isolated by	Type		Arabinose	Mannitol	Sucrose	Lactose	Maltose	Raffinose	Dextrin	Salicin	Aesculin
Orla Jensen	*Thermobacterium**		0	0	?	+	?	0	?	0	?
,,	*Streptobacterium**		?	+	+	+	+	0	?	+	+
,,	*Sbm. casei*†		0	.	0	+	0	0	0	+	+
Ritter	,,		.	+	+	+	+	0	0	0	+
Sadler	,, ‡		0	0	0	+	+	0	0	0	0
J. G. D.	,,	2·11	+	+	+	+	+	+	+	+	+
,,	,,	2·13	+	+	+	+	+	0	+	+	+
,,	,, (intermediate)	2·9	0	+	0	+	+	0	+	+	+
,,	*Sbm. plantarum*	1·4	0	+	+	+	+	0	+	+	+
,,	,,	1·8	0	+	+	+	+	0	?	+	+
,,	,, (weakened)	2·2	0	0	0	0	0	0	0	0	0
Sadler	*Sbm. plantarum*‡		0	0	0	+	+	0	+	0	0
,,	,, ‡		0	0	+	+	+	0	.	+	+
Orla Jensen	Betabacteria*		+	0	?	?	?	?	0	0	0
,,	*Str. lactis*†		0	0	0	+	+	0	+	+	+
J. G. D.	,,		+	+	+	+	+	.	+	+	.
,,	*Str. cremoris*		.	0	0	.	0	.	0	0	.
Orla Jensen	,, †		0	0	0	+	0	.	0	0	0

+ = acidity. ? = irregular fermentation. 0 = no acidity.

* Data bulked from reference (1).

† Cultures obtained from Orla Jensen.

‡ Cultures obtained from the National Collection of Type Cultures.

Str. lactis and *Str. cremoris* and did not give clear-cut turbidity reactions in broth. Similarly certain rod forms resembled *Sbm. plantarum* in morphology but grew fairly rapidly in milk, giving a final acidity of about 1·3 per cent. lactic acid. These strains, at first suspected to be mixed cultures, could not be resolved by streaking and plating and, further, remained constant in their

intermediate properties. They can therefore only be considered as stable intermediate variants, analogous to the intermediate types in the *coli-aerogenes* group. The strains were cultured and frequently examined over a period of two years, during which time no variation from the original description was detected. Moreover, a few rod forms have been cultured for five years without variation.

GENERAL CULTURAL CHARACTERISTICS.

The essential differences between the species having been discussed, it may be mentioned that in all properties the types agreed with those of the corresponding species of Orla Jensen with the single exception that the *Streptobacterium* strains fermented arabinose much more readily than those described by Orla Jensen. It has been found that the ratio of fermentation *rates* of different sugars by species is more characteristic and useful for differentiation than the fermentation or non-fermentation of certain sugars, such as raffinose. In view of the great significance of nitrogen source in sugar fermentation by the lactic acid bacteria, slight differences in sugar fermentation cannot be considered important. Thus, although *Sbm. casei* and *Sbm. plantarum* ferment the same sugars, the latter forms acid from maltose and sucrose faster than from lactose, and from fructose as fast as from dextrose, suggesting its origin from plant sources. *Sbm. casei* ferments lactose as fast as or faster than sucrose and maltose, and dextrose faster than fructose. It would appear to be of greater interest for classification purposes to express the fermentations of sugars in terms of that of dextrose, thus compensating in a convenient way for the differences due to nitrogen source and buffer value of the medium. Further, such a mode of expressing results would at once draw attention to the marked fermentation of certain sugars, such as lactose in the case of the milk lactic acid bacteria, salicin in the case of those saprophytic on animals, and arabinose, fructose, sucrose, maltose and dextrin in the case of those derived from plant material.

"Weakened" strains. Bacteria isolated from older cheese were invariably in a weakened condition and grew more slowly than their corresponding types obtained from less ripe cheese. Indeed, strains of *Sbm. plantarum* from cheese over 6 months old and of *Sbm. casei* from cheese over 12 months old produced barely visible changes in litmus milk after a month and frequently died in subculture. This was apparently due to the loss of the power to ferment lactose. Fig. 2 shows the results of an experiment in which a normally and a slowly growing strain of *Sbm. plantarum* were cultured in broths containing dextrose, galactose and lactose. It may be seen that the normal vigorously growing strain ferments lactose almost as fast as dextrose, whereas the weakened strain ferments dextrose, readily, but lactose only slowly.

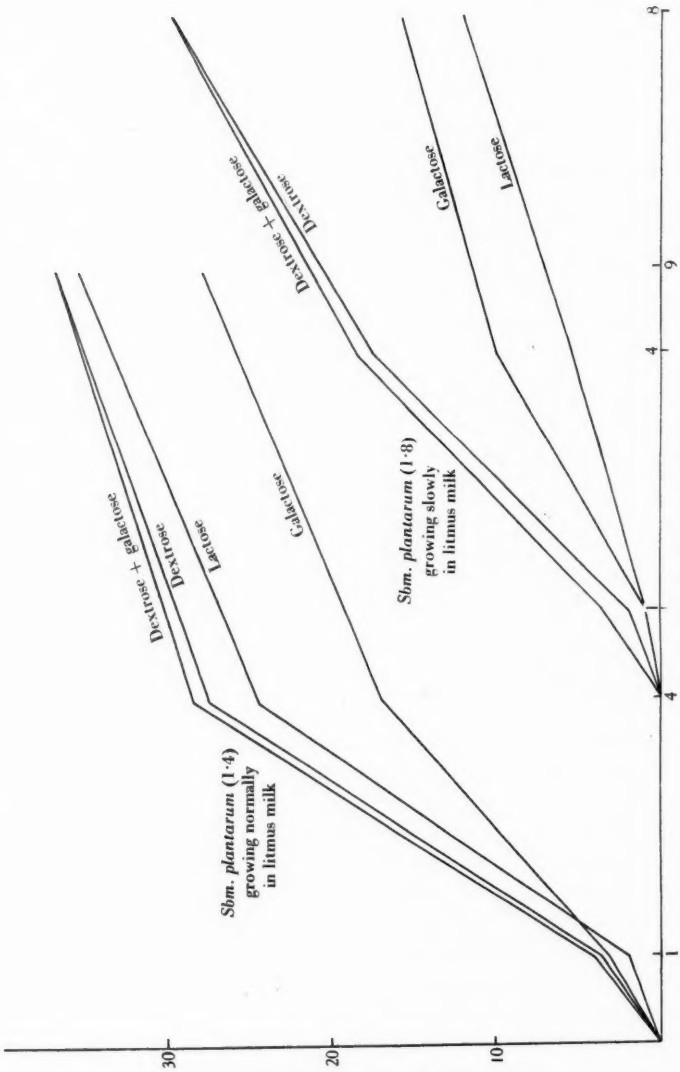


Fig. 2. Sugar fermentation rates by normal and "weakened" strains of *Sbm. plantarum*.

THE FREQUENCY DISTRIBUTION OF BACTERIA IN CHEDDAR CHEESE.

In Table IV are given the numbers of each type isolated from normal or typical Cheddar cheese of different ages. The results are summarised in graphical form in Fig. 3. The cheese were adjudged to be true to type by experienced judges. It is seen that the streptococci die out rapidly after the first month, when the lactobacilli appear. Of the latter the *plantarum* types are found only in the cheese from 1 to 5 months old, after which the *casei* types only remain viable. With both types of lactobacilli, increase in age of the cheese results in an increase in the proportion of "weak" strains, morphologically indistinguishable but giving only slow growth in litmus milk and ultimately dying out on subculture. It is evident that the sequence of bacteria in a ripening cheese is determined by their ability to survive conditions. It is of interest to note that the starters used for making the cheese consisted of *Str. lactis* and *Str. cremoris*. Lactobacilli were never recovered from the starters used.

Table IV. *The frequency distribution of streptococci and lactobacilli in Cheddar cheese made from clean milk.*

		Streptococci			Lactobacilli			
					L. plantarum		*L. casei*	
Age of cheese	Series of isolations	*Str. cremoris*	Intermediates	*Str. lactis*	Normal	Weakened	Normal	Weakened
0 days	5	2	5	8	0	0	0	0
1 day	6	0	1	7	0	0	0	0
3 days	7	11 6*	3	10	0	0	0	0
6 „	13	0	3	4	0	2	1	0
	13†	0	2	3	0	1	4	0
10 „	8	3 2*	2	5	0	11	0	0
22 „	9	12 1*	2	3	0	2	0	0
30 „	10	6	2	5	0	0	0	0
31 „	1	0	3‡	1	6	3	0	0
36 „	18	0	2‡	0	0	3	0	0
40 „	15	1	0	0	0	0	8	0
	15†	0	0	0	0	0	10	0
51 „	17	0	1	0	0	3	3	0
63 „	11	0	0	0	2	2	5	0
70 „	16	0	1‡	0	1	1	6	0
77 „	12	0	0	0	0	0	12	0
5 months	2	0	0	0	3	6	8	0
8 „	1	0	0	0	0	1	7	0
12 „	1	0	0	0	0	1	5	0
18 „	2	0	0	0	0	0	0	13
	Totals	44	27	46	12	36	69	13
		117 streptococci			130 lactobacilli			

* Larged celled, long chained vigorous strains.

† Mass inoculation of *L. casei* 1·12 with starter.

‡ Not identified.

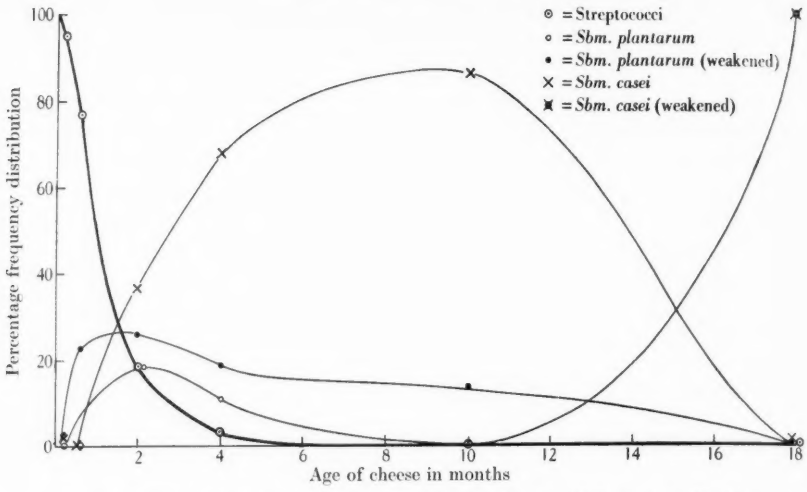


Fig. 3. Curves showing the frequency distribution of lactic acid bacteria in Cheddar cheese at different stages of ripening.

Factors controlling the frequency distribution of bacteria in Cheddar cheese.

Various workers have suggested reasons for the sequence of flora in cheese ripening. These will now be critically considered.

(1) *Titratable acidity.*

This has been shown not to increase appreciably after the first week (3, 4), and is due only in part to free lactic acid. The term is, moreover, a complex one from the point of view of bacterial growth, and its "effect" is best considered under those of its components, such as *p*H, salt concentration, etc.

(2) *Hydrogen-ion concentration.*

The *p*H slowly but definitely increases with the age of the cheese, from about 4·5 at the time of making to about 4·9 after a few days and 5·5 at the end of some months (5). The growth of streptococci is inhibited at about *p*H 4·4, and that of the lactobacilli considered here at about *p*H 3·8. It is generally recognised that streptococci grow best at about *p*H 6·0, and the rod forms at about *p*H 5·5 (6, 7). The *p*H of ripening cheese will to some extent, therefore, favour the lactobacilli.

(3) *Lactate concentration.*

It is recognised that lactates exert an inhibiting influence on the lactic fermentation (8). Experiments on the different susceptibilities of the lactic acid bacteria to sodium lactate have been made by adding lactic acid (neutralised to *p*H 6·6) to dextrose broth to produce various final concentrations and

observing the effect on growth. These experiments are summarised in Table V. It will be observed that all strains of *Str. cremoris* are inhibited by 2 per cent., of *Str. lactis* by 4 per cent., while the rod forms grow quite well with 6 per cent. sodium lactate. This differential inhibition provides a ready method of identification of the main types, and its application for this purpose is under investigation.

In Cheddar cheese after a few days' ripening about 2 per cent. lactic acid is present (or 5 per cent. in the aqueous phase). This exists mostly as combined lactate, and it would appear that lactate concentration is definitely one factor in the inhibition of the streptococci. (*Str. cremoris* does not appear to die out more rapidly than *Str. lactis*, but oxygen tension is here a compensating factor.) It may be observed that the bacteria suffer inhibition in the order of roundness of cell, *Str. cremoris* with roughly spherical cells being most easily

Table V. *The effect of different concentrations of sodium lactate on the growth of lactic acid bacteria in dextrose broth.*

% sodium lactate	2·1		2·9		3·4		3·5		4·1		6·1		8·2	
% dilution of the original dextrose broth	28		42		14		56		70		28		42	
Growth after days	3	20	3	20	3	20	3	20	3	20	3	20	3	20
Str. cremoris Orla Jensen ...	0	0	0	0	0	0	0	0	0	0	0	0	—	—
" 3·4	0	?	0	?	—	—	—	—	—	—	—	—	—	—
" 8·13	—	—	—	—	0	0	—	—	0	0	—	—	—	—
" 13·6	—	—	—	—	—	—	—	—	0	0	—	—	—	—
Str. lactis Orla Jensen ...	?	++	?	+	0	0	0	0	0	0	—	—	—	—
" 1·7	—	—	+	++	—	—	—	—	0	0	—	—	—	—
L. plantarum 1·4	—	—	?	++	+	++	—	—	?	++	?	+	?	+
" 1·8	?	++	?	++	?	+	—	—	?	++	?	+	—	—
L. casei (intermediate) 2·9 ...	+	++	+	++	+	++	—	—	+	++	+	++	—	+
L. casei 1·12	—	—	—	—	+	++	—	—	+	++	+	++	?	+
" 2·13	—	—	++	++	+	++	—	—	+	++	+	++	+	++
" 2·24	?	++	?	++	—	—	—	—	—	—	—	—	—	—

0=no growth. ?=doubtful growth. +=growth. ++=good growth.

inhibited and *Sbm. casei* with long thread-like cells being most resistant. This order is also that of their resistance to acids.

(4) *Nitrogen metabolism of the flora and protein degradation of the cheese.*

Evidence presented in a previous paper(9) has shown that streptococci appear to utilise the intermediate fractions of protein digestion most readily, while the lactobacilli utilise the simpler peptides or amino acids. This metabolic difference may be correlated with the facts that the streptococci grow rapidly in milk (especially when rennet is present), while the lactobacilli grow only slowly. The rates of growth in dextrose broth are of the same order, so that oxygen tension cannot be responsible for this difference. When the protein is broken down, as in peptic digestion, some factor is produced which progressively inhibits the streptococci. The lactobacilli, however, grow faster with increasing digestion of the protein, and this may well be one reason why they gradually supplant the streptococci in cheese. The autolysed cell contents

of the streptococci would provide an admirable medium for the lactobacilli, and may themselves contribute to the non-protein nitrogen figures. Unfortunately it is impossible to determine the *total* bacterial population of a cheese from manufacture to the end of ripening, as the cells are constantly growing and autolysing. Thus no estimate of this fraction can be made.

(5) *Oxidation-reduction potential of the cheese.*

The lactobacilli are commonly regarded as less tolerant of oxygen than the streptococci, but not as strict anaerobes like the *Clostridia*. Their growth in cheese and other milk products after the gradual dying out of the streptococci has been ascribed to the fact that the latter consume all the oxygen and so prepare the correct anaerobic conditions for the former. Interpreted in terms of oxidation-reduction potential, the streptococci can initiate growth under aerobic conditions (*e.g.* at an E_h of plus 0·2 volt), whereas the rods cannot, or do so only with difficulty. This implies that the lactobacilli require the medium to be at a certain minimal potential before their typical metabolic processes can be initiated. As far as Cheddar cheese is concerned, however, both sphere and rod forms appear to possess the same respiratory mechanisms. Both the streptococci (10) and the streptobacteria (11) are quite unable to oxidise sugars in the presence of molecular oxygen or methylene blue, and the very small oxygen consumption detectable by micromanometric methods can be attributed only to intracellular oxidation of tissue substance. Hydrogen peroxide is not produced. In addition, as has already been mentioned, both species grow almost equally well in dextrose broth under aerobic conditions. It may be assumed, therefore, that both species are equally indifferent to (or affected by) oxygen, and that conditions of oxygen tension cannot be responsible for the growth or dying out of either species.

(6) *Carbon sources for growth.*

It has been suggested elsewhere that the lactobacilli, multiplying after the streptococci begin to die (possibly because of the exhaustion of the sugar), are able to grow by utilising lactate as a source of carbon. All attempts to grow the streptococci and lactobacilli in meat extract broth, either alone or with lactates and the salts of a number of other organic acids known to occur in cheese, have failed. Apparent growth in such media has been shown to be due to the traces of sugar carried over with the inoculum, subsequent transplants dying out. Further, such a hypothesis does not agree with the unanimous finding of all investigators that these bacteria must obtain their energy by carbohydrate fermentation. They are known to grow well in protein-rich media containing traces of fermentable sugar (12). Recent work on the presence of traces of sugars in proteins suggests this as a likely source of carbohydrate for the lactobacilli. Thus purified casein has been shown to contain 0·31 per cent. galactose (13).

Although the ordinary tests for sugars in cheese show a complete absence

of these substances after about 10 days, it may safely be assumed that, as the sugar is liberated from the protein by enzymic splitting, it will be fermented by the bacteria present faster than it is set free. The sugar will, therefore, never be detectable by ordinary tests in the presence of strongly saccharolytic bacteria. To prove that sugar, as such, is actually set free in the cheese-ripening process would be a difficult problem. Some slight evidence of this possible liberation of sugar is afforded by the observation that, under certain conditions, a humin-like substance is formed when a cheese mush is being steam distilled for the determination of volatile acids (14). It is established that the formation of humin in protein digests is due to the condensation of carbohydrates with certain amino acids, particularly tryptophane (15).

(7) *Moisture content and osmotic pressure.*

The free water content of cheese decreases continuously, due to evaporation and protein hydrolysis, finally reaching a value of about 35 per cent. It is known that this level is one at which bacteria do not thrive, and it is therefore probable that the lactobacilli, being more resistant generally, are comparatively favoured by the high solids concentration in the cheese. Similarly the osmotic pressure, which must be greater than that of 5 per cent. sodium chloride on account of the salt added (1 oz. to 3 lb. curd), has been shown to affect the streptococci somewhat more than the lactobacilli (1).

(8) *Distance beneath the surface of the cheese.*

While *Str. lactis* and *Str. cremoris* appear to maintain their viability equally well when the whole cheese is considered, a marked inequality in their distribution was found when samples from just beneath the surface and from the centre of the cheese were plated. It may be seen in Table VI that the average ratio of *Str. lactis* to *Str. cremoris* is 2 at a distance of about $\frac{1}{2}$ in. beneath the coat of the cheese, whereas there are five times as many *Str. cremoris* as *Str. lactis* in the depth of the cheese.

By the calculation of χ^2 from the data in Table VI it is found that this

Table VI. *The frequency distribution of* Str. lactis *and* Str. cremoris *near the surface and in the depth of Cheddar cheese.*

		"Surface"* distribution		"Depth"† distribution	
Age of cheese	Date made	*Lactis*	*Cremoris*	*Lactis*	*Cremoris*
3 days	March 1932	7	4	3	14
10 ,,	,,	3	1	2	4
22 ,,	,,	3	4	0	9
30 ,,	,,	5	0	1	6
	Total	18	9	6	33

* About $\frac{1}{2}$ in. from the surface. † About 4 in. from the surface.

coefficient has a value of about 18. Hence P is much less than 0·01 at $n=1$, and the differences in distribution are statistically very significant. This may be considered as evidence that *Str. cremoris* is less tolerant of oxygen than *Str. lactis* (cf. ref. (5)).

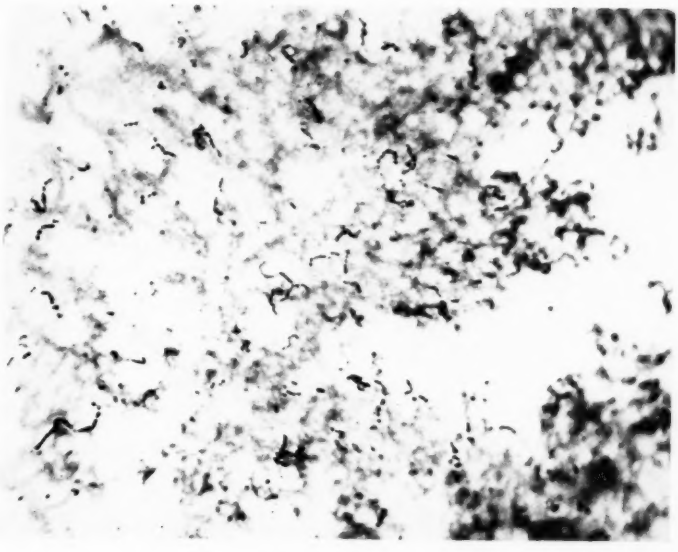
Abnormal types of *Str. cremoris*.

It has already been stated that strains of *Str. cremoris* differ more from strain to strain in their general vigour of growth than they do from *Str. lactis*. A few of the strains isolated were exceedingly vigorous in their growth when transplanted to litmus milk, giving peptonisation and gas production in a few days, and inducing a significantly lower final *p*H in the milk culture. These strains were found in the depth of cheese a few days old and grew well when streaked on dextrose agar plates, in marked distinction from normal strains which gave only scanty surface growth. Such strains always formed very long chains of comparatively gigantic cells (Plate I) of characteristic morphology. They gave the same sugar reactions as normal strains, however, and on subculture returned to normal growth in litmus milk and were no longer morphologically distinguishable. The correlation between increased peptonisation and increased size of cell is in accord with that theory of cheese ripening which assumes the bacterial role to be due to endogenous enzymes. It is known that cheese ripen more rapidly in the depth than near the surface. The correlation between peptonising power and final *p*H in litmus milk is shown in Table VII. Since these abnormally vigorous strains were isolated from freshly made cheese (3–22 days old), it would appear possible that they owe their vigour to the combined effects of rennet and the anaerobic conditions in the green cheese. It is known that the most intensely reducing conditions in Cheddar cheese occur during the first week (5), and that oxidation-reduction potential is an important factor in proteolysis (16).

Although markedly proteolytic, the strains bore no resemblance to *Str. liquefaciens* which dissolves the casein and ferments considerably more sugars than *Str. cremoris*.

Table VII. *Final p*H *values of streptococci.*

Strain	*p*H	
		Strains peptonising milk in 3 days.
7·8	4·35	Very long chains—giant cells—*cremoris* morphology.
7·28	4·37	Average final *p*H = 4·35
7·36	4·35	
8·13	4·33	
8·16	4·33	
		Strains forming gas in 3 days and peptonising in 7 days in milk.
7·21	4·35	Long chains—large cells—*cremoris* morphology.
7·31	4·32	Average final *p*H = 4·36
7·37	4·37	
7·38	4·42	
7·40	4·36	
		Strains peptonising milk in 7 days.
7·3	4·58	*Lactis* type. Average final *p*H = 4·62
7·17	4·55	
7·27	4·66	
7·32	4·68	
7·18	4·63	Intermediate type. Average final *p*H = 4·66
7·23	4·65	
8·19	4·70	



S. cremoris (Orla Jensen)—normal strain (litmus milk culture 2–3 days old).

S. cremoris—vigorous strain inducing low final *p*H and peptonising milk in 3 days (litmus milk culture 2–3 days old).

Table VII (*continued*).

Strain	*p*H	
	Strains peptonising milk in 7 days.	
7·4	4·58	*Cremoris* type. Average final *p*H = 4·53
7·7	4·58	
7·14	4·47	
7·3	4·45	
7·34	4·58	
	Strains not peptonising milk in 10 days.	
6·3	4·61	*Lactis* type. Average final *p*H = 4·59
6·5	4·64	
6·6	4·64	
6·12	4·64	
6·13	4·63	
6·15	4·61	
6·19	4·42	
7·5	4·43	
7·9	4·52	
7·13	4·64	
7·25	4·44	
8·6	4·70	
8·7	4·58	
8·8	4·48	
8·14	4·77	
8·15	4·73	
5·3	4·60	Intermediate type. Average final *p*H = 4·52
5·6	4·55	
5·8	4·53	
5·11	4·44	
5·16	4·63	
6·7	4·53	
8·9	4·39	
7·22	4·57	*Cremoris* type. Average final *p*H = 4·54
7·24	4·42	
7·39	4·50	
8·2	4·52	
8·11	4·68	
8·22	4·54	

SUMMARY.

1. The lactic acid flora of Cheddar cheese made from milk of certified quality form a well-defined, physiologically homogeneous group of bacteria, growing best over a temperature range of from 22 to 37° C. They may be classified into four well-defined types, *Str. lactis*, *Str. cremoris*, *Sbm. plantarum* and *Sbm. casei*, and have been studied over a period of five years. It appears from the evidence found that *Str. lactis* and *Str. cremoris* are distinct species, but that *Sbm. casei* and *Sbm. plantarum* represent different stages in the adaptation of a common progenitor to conditions in a ripening cheese. Both the streptococci and the streptobacteria appear to be unable to oxidise sugars and may thus be considered indifferent to molecular oxygen.

2. A study of their frequency distribution from the curd at making to an 18 months old cheese has shown that *Str. lactis* and *Str. cremoris* are equally viable during the first month, after which the rod forms begin to predominate, *Sbm. plantarum* and, later, *Sbm. casei* being found. The former lactobacillus is only found when the cheese is from 1 to 5 months old, the flora con-

sisting entirely of *Sbm. casei* after this time. The general vigour of all strains decreases with increasing age of the cheese. There is a marked correlation between the shape of the cell, the viability of the organism in cheese and its resistance to acids and lactates.

3. The factors controlling the sequence of flora in Cheddar cheese are discussed. There is no evidence that titratable acidity, oxygen tension and differential carbon sources are responsible for the sequence. It is suggested that lactate concentration, the extent of protein degradation and osmotic pressure are factors responsible for the gradual replacement of the streptococci by the rod forms.

4. The significance of sugar fermentations by the lactic acid bacteria studied is discussed. The slow production of lactase is shown to be the reason for the slow growth of weakened strains in litmus milk.

5. *Str. cremoris* predominates over *Str. lactis* in the depth of the cheese in the early stages of ripening, whereas near the surface the reverse holds. Certain strains of *Str. cremoris* isolated from the depth of the cheese were particularly vigorous in growth in litmus milk, forming gas and beginning to peptonise the milk in about 3 days. Such strains consisted of very long chains of large cells of peculiar morphology. It is suggested that this finding is related to the known greater rate of ripening in the depth of the cheese.

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105. THE PHOSPHATASE TEST FOR PASTEURISED MILK

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(With 1 Figure.)

The temperature and duration of heating to which milk must be submitted if it is properly to be described as pasteurised have been legally defined in many countries. In Great Britain the stipulations are that the milk must be kept for 30 min. at a temperature between 145 and 150° F. and immediately cooled to 55° F. In Canada and some parts of the United States the temperature is from 140 to 145° F., the time of exposure being the same.[1]

Although the British regulations provide a margin of safety which is somewhat greater than is provided by the regulations of certain of the United States and of Canada, they were not arrived at without much thought and experiment, and presumably, expected to be strictly obeyed. Our own experience on both sides of the Atlantic has convinced us that although some of the distributors are doing the work of pasteurisation with consistent efficiency, and in some towns the supervision exercised by the authorities leaves nothing to be desired, yet nevertheless much of the milk on the market labelled and described as "pasteurised" is, in fact, nothing of the kind. In view of the large quantity of raw milk contaminated with *Myco. tuberculosis* which arrives daily in urban centres, the great importance from the public health point of view of devising methods for the proper diagnosis of incorrectly performed pasteurisation need not be laboured.

Various types and makes of pasteurising plant in use to-day require very varying amounts of care and attention before they will consistently produce a satisfactorily treated article. With many of these plants, the methods which have been used up to the present to control efficiency of pasteurisation have been too clumsy to do other than slightly diminish the rather large loopholes for faulty manipulation and negligence. It is true that in some instances the construction and detailed functioning of a pasteurising plant are thoroughly examined mechanically and bacteriologically by the responsible authority. Surprise visits are sometimes paid and temperature recorders and records are, not infrequently, carefully scrutinised.

[1] The U.S. Department of Agriculture defines pasteurised milk as having been "subjected to a temperature not lower than 145° F. for not less than 30 minutes...". The U. S. Public Health Service accepts "a temperature not less than 142° F. and holding at such temperature for not less than 30 minutes..." with certain important stipulations as to thermometer temperature tolerance.

The results of an examination of the alleged pasteurised milk for the presence of *Bact. coli* are also, on occasions, taken into account. These organisms, as is well known, should not be present in appreciable numbers in properly pasteurised milk immediately after pasteurisation.

No one of these methods is, by itself, satisfactory. Of those just mentioned, the first three require repeated visits to the plant, are time-consuming and are neither fool-proof nor knave-proof. Examination of the final product at frequent intervals is clearly the most satisfactory method, if a suitable test can be devised. Until recently there was no satisfactory test which could be applied *to the product* to assess the efficiency with which a given milk had been pasteurised, as has been pointed out by Zeller.(1)

The heating of milk, whether done commercially merely to improve its keeping quality or done to comply with public health requirements for pasteurisation, inactivates more or less completely the enzymes which were originally present in the raw milk. The completeness of their destruction obviously depends very largely on the temperature and the time during which the milk has been exposed to heat, and on the specific characteristics of the individual enzymes, some, like peroxidase, being more thermostable than others, such as amylase (Orla-Jensen(2)).

A method for distinguishing between raw and pasteurised milk which depended on whether the enzyme phosphatase (which is a constant constituent of raw milk) had or had not been destroyed was recently described in this *Journal* (Kay and Graham(3)). Two different tests were outlined, a *quantitative* method for determining, in arbitrary units, the amount of phosphatase present and a quick, but relatively rough *qualitative* test for determining whether a given sample of milk had or had not been heated.

Although both these tests were found useful in our own and in other laboratories, we were by no means satisfied that further experiment would not yield us methods, based upon the heat instability of phosphatase, which would be more searching and informative than those we had already suggested. In the present communication the development of the two original tests into weapons of greater precision will be described. Considerable modifications have in fact been made. Substrate, buffer, time and temperature have all been carefully examined and have been revised, together with the method of determining the enzyme quantitatively. As a result of the accumulation of several important modifications, the technique now differs considerably from the original. Of the revised tests the first, test A, gives much greater speed (the whole test can now be completed in $\frac{1}{2}$ hour) and a little greater accuracy, and with the other, test B, much greater accuracy. The definiteness with which efficient pasteurisation can now be displayed, and the certainty with which faulty methods of heat treatment can be revealed, encourage us to present the revised tests in detail.

The principle remains the same, *i.e.* (1) the addition of small portions of the milk under test to large excess of a phosphoric ester, (2) a standard period of

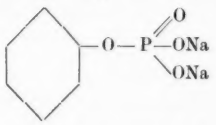
incubation under standard conditions during which phosphatase, if present in the milk, will hydrolyse the ester (which is otherwise quite stable), (3) the stopping of the hydrolysis after a given time followed by the colorimetric determination of the end-products.

The phosphoric ester used as substrate (formerly this was sodium β-glycerophosphate) is now disodium phenyl phosphate which is hydrolysed by phosphatase, under the conditions of the test about twice as fast as is the glycerophosphate (King and Armstrong (4)). Moreover, instead of determining the inorganic phosphate liberated by the active enzyme we find, as recommended by King and Armstrong, that it is more convenient and delicate to determine the free phenol, using the Folin colorimetric phenol reagent[1] (Folin and Ciocalteu (5)), which does not react with phenyl phosphate, with considerable gain in the amount of colour produced for unit quantity of substrate hydrolysed. As buffer we now use sodium diethyl barbiturate ("sodium veronal") which Michaelis (6) has shown to be an excellent buffer within the *p*H range with which we are concerned. For assessing the colour produced, either a modified but very simple comparator, or the improved Lovibond tintometer, or a precision colorimeter, may be used.

DESCRIPTION OF METHODS.

(*a*) *Reagents required.*

(1) *Buffer substrate.* Dissolve 1·09 g. of disodium phenyl phosphate[2] and



11·54 g. of "sodium veronal"[2] in water saturated with chloroform and make up to 1 litre. Add a few drops of chloroform to prevent the growth of micro-organisms, and keep in the refrigerator.

(2) *Folin and Ciocalteu's phenol reagent.*[2] Dissolve 100 g. of sodium tungstate $Na_2WO_4.2H_2O$ and 25 g. of sodium molybdate, $Na_2MoO_4.2H_2O$, in 700 ml. of water in a 1500 ml. flask connected, preferably by ground glass joint, with a reflux condenser. Add 50 ml. syrupy (85 per cent.) phosphoric acid and 100 ml. of concentrated hydrochloric acid. Reflux the mixture gently for 10 hours. (If an all-glass connection between flask and condenser is not available, use a rubber stopper or cork wrapped in tin foil. Take the greatest care that the solution does not come in contact with the tin foil.)

[1] An attempt was made to use *p*-nitraniline instead of Folin's reagent for estimating the phenol. The *p*-nitraniline reagent is much more easily prepared than Folin's reagent, and the colour given is definite and stable, but we found the red *p*-nitraniline colour less easily detectable and measurable in the important zone of not-quite-satisfactory pasteurisation, particularly in test A (p. 194).

[2] Obtainable from British Drug Houses, Ltd.

After 10 hours, cool, add 150 g. pure lithium sulphate, 50 ml. water and a few drops (usually 4–6) of liquid bromine. Boil the mixture under the hood without the condenser for 15 min. to boil off excess of bromine. Cool, dilute to 1 litre and filter. The finished reagent should have a golden yellow colour with no greenish tint. Any reagent with a greenish tint should be rejected. Keep well protected from dust. Dilute 1 volume of this stock solution with 2 volumes of water before use.

(3) A 14 per cent. solution of anhydrous sodium carbonate Na_2CO_3 (analytical reagent quality).[1]

(*b*) *Special apparatus required.*

(1) A Lovibond tintometer or, for most purposes, a simple comparator (see below) with Lovibond standard cell.

(2) Whatman No. 30 filter papers, 9 cm. (or acid-washed filter papers of similar quality).

(3) A water bath which can be kept at 47° C. for a short time (10 min.).

(4) An incubator maintained at 37–38° C.

Other than these all that is required is a good supply of fairly stout test-tubes of constant diameter ($\frac{5}{8}$ in.) and stoppers of small filter funnels to take the 9 cm. paper comfortably, and a beaker of boiling water.

(*c*) *Technique of short test* (*test* A).

(1) Run 10 ml. of substrate-buffer solution ((*a*) (1) above) into each of four test-tubes of capacity 20–25 ml. To two tubes (control tubes) add 4·5 ml. of diluted Folin's reagent. To all four tubes add 0·5 ml. of the milk to be tested, mix well. Incubate the two tubes which do *not* contain the Folin's reagent at 47° C. $\pm$ 2° in the water bath for 10 min.

(2) After 10 min. remove the tubes from the water bath, cool, add 4·5 ml. of diluted Folin's reagent. Keep for 3 min. Filter all four tubes.

(3) To 10 ml. of filtrate add 2·0 ml. of 14 per cent. sodium carbonate solution, mix, place in boiling water bath for 5 min. Filter.

If only a faint blue colour develops in all four tubes, the milk has been heated, but not necessarily properly pasteurised. If the controls show more than a trace of blue colour, *i.e.* more than about 1·5 units (see below), the reagents should be carefully looked over, and if these are in good order, with no free phenol in any of them, it is likely that a phenol-producing organism is present in the milk sample. This does *not* occur in pasteurised milk which has been kept at a satisfactorily low temperature since pasteurisation.

With some experience, it will be found possible to omit the control tubes for the great majority of samples submitted to this test. With fresh, properly pasteurised milk, *i.e.* milk within 18 hours of pasteurisation which has been cooled to 55° F. immediately after pasteurisation and maintained between

[1] All the chemical substances used for making up these reagents should be of "analytical reagent" quality.

that temperature and 65° F., the control tubes should show only a trace of blue colour. If with such controls a definite blue colour develops in the *incubated* tubes (*i.e.* a colour greater than the standard colour as described below), it can be said with considerable certainty that the milk has not been adequately pasteurised.

Test A permits one, therefore, to decide within a few minutes whether a given sample of milk has or has not been heated. Gross errors in the prescribed technique for pasteurisation may be recognised quickly by it. To make the test a little more precise, the coloured solution (assuming that the control tube gives no reaction, *i.e.* that the colour in it is no more than a very faint blue) may be compared by pouring direct into a standard 13 mm. cell[1] against a standard glass made to contain 2·3 Lovibond units of blue. Such a piece of glass[2] may readily be incorporated in a small simple two-holed comparator, made to take the standard "acid resistant" cell[2] in one hole, and the coloured glass (50 by 18 mm.) inserted in the other hole. We use this apparatus with the comparator held in an ordinary retort-stand clamp at a few inches distant from a piece of white tile or paper on the bench reflecting light from a window or from a "daylight" lamp through the comparator.

It will be observed that this first test is (*a*) rapid (total time for a single determination 30 min., several dozen may be done in an hour), (*b*) simple (the apparatus and reagents are straightforward and readily obtainable, and although care is needed in making up the solutions and in maintaining cleanliness, a properly supervised laboratory assistant can carry out all the operations with ease), (*c*) certain, *if the test is properly carried out, a colour greater than the standard glass means that the milk has not been properly pasteurised.*

The converse is, however, not true. If the blue colour developed in test A does not exceed the standard, we are *not* certain that the milk has been properly pasteurised. If, for example, the temperature or heating has only been 143° F. instead of 145° F., the first test will probably show little colour.

In case, then, the colour developed in the first test is close to the standard colour, or there is any reason to suspect minor errors of pasteurisation which might not be revealed by the first test, the second, more delicate test may be proceeded with.

(*d*) *Technique of longer test* (*test* B).

(1) Run 10 ml. of buffer-substrate solution ((*a*) (1) above) into each of four test-tubes of capacity 20–25 ml. (If the controls in test A have been negative, test B controls need not be used, and two instead of four test-tubes are all that are required for test B.) To two tubes (control tubes) add 4·5 ml. of diluted Folin's reagent. To all four tubes add 0·5 ml. of milk to be tested. Mix well. Up to this point, the procedure is exactly the same as in the shorter test. Now, however, add 2 drops of chloroform to, and stopper, each of the two tubes in

[1] It is not absolutely necessary to have this cell for the first test; the presence or absence of blue colour is sufficient, without the standard glass.

[2] Obtainable from Tintometer, Ltd., Colour Laboratory, Milford, Salisbury, or from British Drug Houses, Ltd.

which the milk has not been precipitated, warm to 37–38° C. and maintain at this temperature (a properly controlled bacteriological incubator will serve) for 24 hours.

(2) At the end of this time, remove from incubator, add 4·5 ml. of diluted Folin's reagent to each tube, stand for 3 min., filter all four tubes. Add 2·0 ml. of the sodium carbonate solution to 10 ml. of filtrate, mix thoroughly and place in boiling water for 5 min. Filter. The filtrate is placed in the standard cell, and compared with the standard glass as above, or a Lovibond tintometer, if available, may be used. If the controls show only a very faint blue colour, and if the blue colour developed in the tubes after incubation exceeds that of the standard glass (or 2·3 Lovibond units of blue on the tintometer scale), then the milk has been improperly pasteurised, *i.e.* either (*a*) the temperature of

Table I. *Test B carried out on samples of commercial mixed milk pasteurised under "legal" conditions in the laboratory, at* 145° F. *for exactly* 30 *min., then cooled rapidly.*

Serial number of milk sample	Lovibond blue units	Serial number of milk sample	Lovibond blue units
1	2·0	20	2·1
2	1·6	21	1·9
3	1·7	22	2·2
4	1·3	23	1·9
5	1·9	24	1·9
6	1·7	25	1·7
7	1·8	26	2·1
8	1·5	27	2·0
9	1·7	28	1·7
10	1·9	29	1·7
11	1·9	30	1·9
12	1·9	31	2·0
13	1·5	32	1·9
14	2·0	33	1·6
15	1·9	34	1·7
16	1·8	35	1·8
17	1·6	36	1·7
18	1·8	37	2·1
19	2·0	38	1·9

pasteurisation has been too low (see Table II), or (*b*) the duration of heating at the pasteurisation temperature has been too short (see Table III), or (*c*) a small quantity of raw milk has found its way into the pasteurised bulk (see Table IV).

In Table I will be found some typical results obtained by laboratory pasteurisation of mixed milks, using the second test (test B). It will be observed that, pasteurised for 30 min. *at 145°* F. (and tested within a few hours after pasteurisation), no sample gave a colour deeper than 2·3 Lovibond blue units. Other fairly wide experience of this test carried out under the conditions just described has never yielded an example of a properly pasteurised milk giving a colour exceeding this value. 2·3 units has therefore been taken as the maximum colour allowable for properly pasteurised milk. This is probably a little on the generous side. So far all commercial samples which we believe

from other reasons to have been properly and efficiently pasteurised have been well within this limit.

Table II. *Effect of temperature of pasteurisation on development of blue colour in test B. Milks pasteurised at temperatures from* 137·5 *to* 145° F. *for exactly* ½ *hour.*

Serial number of milk sample	Temperature of pasteurisation ° F.			
	137·5	140	142·5	145
	Lovibond blue units			
41*	35·0	19·4	4·4	1·8
42*	33·5	22·8	5·0	2·0
43*	31·0	22·5	4·5	1·8
44*	32·5	21·0	4·6	2·1
45	9·6	3·7	2·3†	1·9
46	30·5	12·2	3·1	1·6
47	28·5	23·7	3·1	1·7
48	12·8	6·4	2·2†	1·8
49	30·6	24·0	3·0	1·8
50	25·2	23·4	4·2	1·9
51	30·4	16·3	3·7	2·3
52	24·0	11·0	2·5	1·6

* Commercial, mixed milk. The other samples were from individual cows of our own herd.

† There is considerable variation in the phosphatase content of the milk from one individual to another. The phosphatase value may be sufficiently low originally in the milk of occasional individual cows (usually in the early stages of lactation) that heating at 142·5° F. is enough to reduce the blue colour to below the standard of 2·3 units. This in our experience does not occur with the mixed milk of a herd.

Table III. *Effect of duration of heating at pasteurisation temperature on development of blue colour in test B.*

Serial number of milk sample	Time in min. milk maintained at pasteurisation temperature of 145° F.			
	10	20	25	30
	Lovibond blue units			
54	16·6	3·3	2·2	1·8
55	7·8	2·5	1·9	1·7
56	23·8	9·0	3·0	2·1
57	24·6	4·9	3·1	1·9

Table IV. *Effect on development of blue colour in test B of addition of small quantities of raw milk to milk pasteurised at* 145° F. *for* 30 *min.*

Amount of raw milk added parts per 100	Milk 61	Milk 62
	Lovibond blue units	
0·00	1·7	1·6
0·05	1·8	1·9
0·10	2·1	2·0
0·15	2·5	2·3
0·20	2·5	2·5
0·25	2·6	2·9
0·50	3·7	4·2

From the figures in Table IV and other findings of a similar kind, it appears possible to detect by the phosphatase test B as little as 0·2 per cent. addition of raw milk, *i.e.* 1 part in 500, to milk pasteurised at 145° F.

Application of phosphatase tests to different conditions of pasteurisation.

(1) *American and Canadian conditions.*

In U.S.A. the standard of pasteurisation varies somewhat from State to State, and in some cases the minimum temperature at which heating by the holder process may be carried out is as low as 140° F. This is true of Canada also, where the range allowed is 140–145° F. Exposure of the milk to such temperatures for ½ hour is generally accepted as being sufficient to destroy the common non-spore-forming pathogenic organisms present, but it is insufficient, particularly in the neighbourhood of 140° F., completely to destroy the phosphatase. Nevertheless, by suitable modifications the phosphatase test can be adjusted to suit the American standards. After some trials it was found that the best method for testing whether milk had been properly pasteurised at temperatures lower than those legal in Great Britain was to keep the same blue unit maximum of 2·3 units for properly pasteurised milk but diminish the length of time of incubation to the appropriate extent. It was found that no milks pasteurised in the laboratory for ½ hour at 140 ± 0·5° F. after incubating at 37° C. for 1 hour[1] instead of 24 hours in test B, gave more than 2·2 blue units. Thus, if a milk which is claimed to have been held at 140° F. or above for ½ hour gives more than 2·3 blue units under these conditions, it has almost certainly been improperly handled.

We have given in Fig. 1 a graph which permits of the length of time of incubation in test B to be adjusted to suit the temperature at which the milk is claimed to have been held. We have tested a large number of milks, pasteurised at various temperatures (between 137·5 and 150° F.) for ½ hour, and then incubated, under test B conditions, for periods varying from ½ to 24 hours. On a basis of some three hundred determinations the line shown in Fig. 1 has been drawn to show the minimum time during which it is possible to incubate pasteurised milk with the phosphoric ester, under test B conditions at 37° C., without the production of more than the standard colour of 2·3 blue units; *e.g.* if any given milk has been claimed to be heated for ½ hour say at 142–143° F., it should not give more than 2·3 units after 2½ hours' incubation at 37° C. If it gives a greater colour than this after 2½ hours' incubation it has not been heated as claimed. The line in Fig. 1 is drawn rather generously; actually all mixed milks should permit of a somewhat greater length of time of incubation without showing 2·3 units of blue with test B. The line shows the *minimum* limit of time.

(2) *Milk subjected to high-temperature, short-time process.*

We have carried out experiments on samples of milk from fourteen different runs of a plant in which milk was heated by the high-temperature short-time process recommended by Stassano. The milk was maintained at 167 ± 0·5° F. for a period believed to be between 15 and 20 sec., and then

[1] A number of such milks when incubated for 1½ hours gave blue colours greater than 2·3 units.

cooled rapidly to 40–45° F. The tests A and B were uniformly negative. The milk before this treatment gave every evidence of being raw, with a strong phosphatase reaction. After the heating the enzyme had been completely destroyed, and therefore, presumably, pathogenic organisms also.[1] (If North and Park's careful experiments be accepted as valid, pathogenic organisms appear to be more destructible over all ranges of temperature-time relationships examined than the enzyme (Kay and Graham (3)).

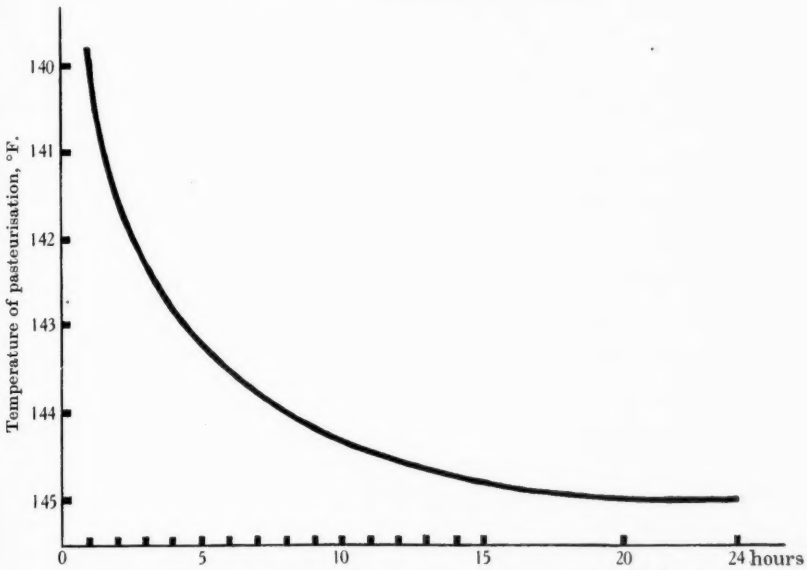


Fig. 1. Minimum time of incubation of properly pasteurised milk with the phosphoric ester at 37–38° C.

Various methods of determining whether or not a milk has exceeded the standard colour in tests A and B.

For test A, after a little experience, the eye without extraneous help in the way of standard colours is good enough for the majority of samples tested. At the beginning, until experience is gained, the test may be controlled either by the Lovibond tintometer or by the rather simpler comparator device.

For test B it is essential to use a colour standard, either the simple comparator with the Lovibond cell and special glass standard, or the Lovibond tintometer, or a colorimeter. If the last of these is used, it is most convenient to make up a standard colour as follows:

Prepare a solution of pure phenol in 0·1 *N* HCl to contain approximately

[1] This implies an assumption that we are not, perhaps, entitled to make, either with this or with any other type of pasteurisation plant, that there have been no small leaks nor inadvertent mixing with raw milk, in quantities insufficient to give the phosphatase test.

1 mg. of crystalline phenol per ml. Transfer 25 ml. of this solution to a 250 ml. flask, add 50 ml. of 0·1 *N* NaOH and heat to 65° C. To the hot solution add 25 ml. of 0·1 *N* iodine solution, stopper the flask and stand at room temperature for 30–40 min. Then add 5 ml. of concentrated HCl, and titrate the excess iodine with 0·1 *N* thiosulphate solution. Each ml. of 0·1 *N* iodine (ml. of iodine added minus ml. of thiosulphate used for back titration) corresponds with 1·567 mg. phenol. On the basis of this titration the original phenol solution should be diluted to contain 0·1 mg. phenol per ml.

For preparation of the standard colour, add 1 ml. of the adjusted phenol solution to 40 ml. of a mixture of 10 parts of the buffer-substrate solution and 4·5 parts of the diluted Folin's reagent. To 10 ml. of this mixture add 2 ml. of the 14 per cent. sodium carbonate solution, place in boiling water bath for 5 min.

This standard colour can be used in a comparator, or colorimeter, or, almost equally well, in similar test-tubes in a test-tube rack to compare with the colour given by the milk under test.

Application of phosphatase test to commercially pasteurised milks.

In Table V are given the results given by tests A and B when carried out on commercially pasteurised milks (either designated pasteurised, or sold to school children as pasteurised, under the "Milk in Schools" scheme).

The results shown in the table comprise all the stated pasteurised milks which arrived in our laboratory over a period of about six weeks. They are not selected. It will be noticed that about one-quarter of the designated, and one-half of the school children's milks are either raw or improperly pasteurised.

Application of the phosphatase test to butter.

Take about 30 g. of butter in 100 ml. centrifuge tube, melt at 40° C. and centrifuge. Pipette off the aqueous layer.

In test A, substitute ½ ml. of this aqueous layer for the ½ ml. of milk. Heat for 10 min. at 47° C., cool, develop colour as in test A. A blue of greater than the standard 2·3 blue units suggests a raw or poorly pasteurised cream. This has been confirmed with butter made from raw cream and from cream pasteurised in the laboratory by the holder process at 145° C. for ½ hour, and with butter made in New Zealand from raw cream and from cream pasteurised by the flash pasteurisation method in common use there. Further experiments are in progress on the application of the two tests to butter.

Difficulties.

Some of the difficulties that may be encountered when taking up for the first time the methods just described are as follows:

(1) *Folin's reagent.* This must be carefully made up according to the method given above, which is practically the same as the original method described by Folin and Ciocalteu (1927).

(2) *Stability of phenyl phosphate.* This reagent should be made up every three days, kept in the cold, and a few drops of chloroform should be shaken into the solution to check the growth of micro-organisms.

(3) *Filter papers.* A good, acid-washed paper should be used. We have found Whatman No. 30 satisfactory.

Table V. *Results of tests A and B carried out on samples of milk stated to be pasteurised.*

Sample No.	How obtained	Results of test A	Results of test B	Remarks
	Milks designated pasteurised			
71*	Bottle from roundsman	–	–	Properly heated
72*	Bottle from roundsman	–	–	Properly heated
73	Official sample	– ?	+	Heated, but not properly pasteurised
74	Official sample	–	–	Properly heated
75	Official sample	+	+	Probably raw
76	Official sample	–	–	Properly heated
77*	Bottle from roundsman	–	–	Properly heated
78	Bottle from roundsman	–	–	Properly heated
79	Official sample	–	–	Properly heated
80	Official sample (from cooler)	–	–	Properly heated
81	Official sample	–	–	Properly heated
82	Official sample	+	+	Poorly heated
83	Official sample	+	+	? Raw
84*	Bottle from roundsman	–	–	Properly heated
85*	Bottle from roundsman	–	–	Properly heated
	School milk given to children as pasteurised (all designated except 5s and 18s)			
1s	Bottle from school	–	–	Properly heated
2s	Bottle from school	+	+	Probably raw
3s	From churn	–	–	Properly heated
4s	Bottle from school	+ ?	+	Some heat treatment
5s	Bottle from school	+	+	Probably raw
6s	From churn	+	+	Probably raw
7s*	Bottle from school	–	–	Properly heated
8s*	Bottle from school	–	–	Properly heated
9s	Official sample	+	+	Probably raw
10s	Official sample	–	–	Properly heated
11s	Official sample	+	+	Probably raw
12s	Official sample	–	–	Properly heated
13s	Official sample	–	–	Properly heated
14s	Bottle from school	–	–	Properly heated
15s	Official sample	–	–	Properly heated
16s	Official sample	– ?	+	Had received some heat treatment
17s	Bottle from school	– ?	+	
18s	Bottle from school	– ?	+	
19s	Bottle from school	–	–	Properly heated
20s	Bottle from school	+	+	Probably raw

* Pasteurisation plants known to be under careful supervision.

(4) *Abnormal milk.* The milk of an individual cow in an early stage of lactation may have a fairly low phosphatase content (as low as one-fifth of that of an average mixed herd sample). This should be borne in mind if tests of milk from individual cows are being made. For mixed milk samples this difficulty is very unlikely to arise.

(5) *Phenol-producing organisms.* Very occasional anomalies may occur in milks where phenol-producing organisms have become active. In such milks a blue colour may be given by the milk as received, without any incubation

period at all, and also of course by such milks even when properly pasteurised in the laboratory just before the test. It is unlikely to occur in actual practice, though in one or two samples of pasteurised milk which had been kept at a temperature of 65° F. or above for several hours, small amounts of phenol have been observed. This risk may be eliminated by using control tubes as indicated under "Technique of tests".

(6) *Phenol in the laboratory.* It need hardly be mentioned that care must be taken to keep phenols, disinfectants containing phenols and soap containing carbolic acid at a safe distance from the test reagents and apparatus used.

Discussion.

Any test for efficient pasteurisation which is to be of value in a milk control or a public health laboratory must fulfil as many as possible of the following requirements: (*a*) It should be quick, (*b*) it should be capable of being carried out on small, easily portable samples, (*c*) it should be reasonably simple, (*d*) it should be cheap, (*e*) it should be certain, (*f*) it should be capable of detecting small as well as large deficiencies below the legal standards, (*g*) it should be capable of quantitative expression, (*h*) if possible it ought to have some known relationship to the thermal death rate of pathogenic organisms, (*i*) it should, if possible, be applicable to heated cream and butter and other products made from heated cream.

No single test devised up to the present fulfils all these requirements, but our experience with the two phosphatase tests just described lead us to believe that, taken together, they will in fact do so.

Test A will tell us, in a few minutes, whether a given sample of milk has been heated sufficiently to destroy pathogenic organisms. If it is negative, then the milk has undoubtedly been heated, and in the light of Fig. 2 of our 1933 paper (Kay and Graham (3)) the only possibilities of pathogenic organisms being present would be either if raw unheated milk (and that in very small amounts, or test A would detect it) had entered the main bulk after pasteurisation, but before sampling, or if organisms had got into the milk *after the heating process* in some other way—a rather rare contingency and one that pasteurisation in any case would not avoid. Thus it may ultimately be found to be waste of time and animals to carry out the biological test for the presence of tubercle bacilli (guinea-pig test) on milk that gives a negative test A.

Test B may be applied to milks which are on the borderline of test A, or where it is suspected that the temperature or duration of holding are somewhat below the required standards. Test B can be used quantitatively (by estimation of the blue units produced under standard conditions), and if carried out with due care will reveal quite small deficiencies of pasteurisation technique. A small deficiency suggests the desirability of an advisory letter or visit. A large reaction, by either test A or test B, means, with any sample of milk alleged to be pasteurised, gross inefficiency. A large reaction in urban pasteurised milk

indicates, in view of the almost universal presence of *Myco. tuberculosis* in the mixed bulked milk arriving in tanks at most urban pasteurising plants, that living pathogenic organisms may be getting through into the milk which is being placed in the consumers' hands as pasteurised. A guinea-pig test, which is hardly worth while carrying out on milks negative to test A or test B, will, if carried out on positive "pasteurised" samples undoubtedly reveal that a number of such milks contain the tubercle bacillus.

SUMMARY.

Details are given of two simple tests for efficiency of pasteurisation. The first, test A, may be carried out completely within half an hour, and will detect any gross errors of pasteurisation technique. The second, test B, is more searching, and will reveal minor errors of technique as follows:

(*a*) $1\frac{1}{2}°$ F. below the minimum temperature for pasteurisation, or (*b*) heating (at 145° F.) for 20 instead of 30 min., or (*c*) admixture of 0·25 per cent. of raw milk with properly pasteurised milk. Taken together, the two tests form an efficient method for the laboratory diagnosis of faulty pasteurisation.

We are much indebted to Dr E. J. King for mentioning personally to us, in advance of publication, the valuable modification introduced by Armstrong and himself of the older methods of determining phosphatase in blood serum, to Prof. A. Riddet for samples of butter prepared under carefully controlled conditions in New Zealand, to Messrs J. McClemont and W. A. Hoy for samples of commercially pasteurised milks, to Capt. W. A. Nell and Mr A. L. Barton for samples of milk subjected to Stassano's process and to Mr F. K. Neave for assistance in the experimental work.

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106. THE ROLE OF RENNET IN THE RIPENING OF CHEDDAR CHEESE

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(With 6 Figures.)

Numerous investigations upon the ripening of Cheddar cheese have failed to correlate the characteristic flavour with the presence of specific chemical compounds. Any such substances would be expected to originate in protein, since it is the protein which undergoes by far the greatest change during ripening. From analogy with the important role of micro-organisms in several other varieties of cheese, and also because of the fact that cheesy odours have been produced in certain media by bacterial action, it is generally assumed that the causative agent in Cheddar cheese is the lactic acid group of bacteria. These views, which lack rigid proof, are lent support by the frequently quoted statement that, in the absence of micro-organisms, flavour does not develop. For evidence upon this point, Van Slyke and collaborators (1), extending the earlier fragmentary work of Adametz (2), relied mainly upon the preparation of cheese from chloroformed milk. Unfortunately no details appear to be given as to how it was decided that flavour was absent from the cheeses containing chloroform.

In the cheese analysed by Van Slyke very large differences were observed in the mode of protein degradation, both qualitatively and quantitatively, from that occurring in normal cheese. It appeared as though the rennet enzyme played a relatively minor part in the breakdown, and a considerable proportion of the change during cheese-ripening was ascribed to the action of other agents, presumably bacterial enzymes. It is obvious, however, that a most serious defect in these experiments lay in the complete lack of acid development in the chloroformed vats during the process of manufacture; for not only does the formation of acid influence the proportion of mineral constituents which remain in the curd, but it also governs the formation of calcium lactate, calcium acid phosphate and monocalcium paracaseinate. In other words, the whole nature of the curd is altered as a substrate for the subsequent action of enzymes. Furthermore, the final acidity of the curd during the making process determines to a marked degree the rate of protein degradation brought about by the rennet. These two factors alone would account for a considerable difference between normal cheese and those prepared from chloroformed milk. As will be shown in the present paper, the retarding effect of chloroform on the action of the proteolytic enzyme in rennet is yet another

important factor in accentuating the difference between normal and chloroformed cheeses as prepared by Van Slyke and co-workers (3, 4).

The importance of rennet and galactase as proteolytic agents in cheese was early emphasised by Babcock and co-workers (5, 6), but much confusion still exists in the quantitative apportioning of their function. The role of micro-organisms in protein breakdown is a much debated point (the well-known views of Barthel and collaborators have been discussed briefly in a recent communication (7)), and no satisfactory measure has been established for the proteolysis brought about in Cheddar cheese by bacteria.

In the present investigation chloroform was employed as a means of eliminating the bacterial factor; and an assessment of the part played by rennet in cheese-ripening was thus rendered possible. The effect of chloroform upon the proteolytic action of rennet in milk having been examined, the results were applied in a study of the ripening of cheese in the presence of this germicide. Preliminary tests, in which small samples of cheese admixed with chloroform were analysed after a ripening period, were followed by a more complete investigation of cheeses chloroformed at the salting stage of the manufacturing process. In the final series of experiments the study was extended to cheese treated with chloroform in the early stages of ripening.

Methods of analysis. Analyses were carried out employing the modified technique of Wasteneys and Borsook described in an earlier paper (7).

I. The influence of chloroform and toluene upon the digestion of milk by rennet and trypsin.

It was intended, as a matter of interest, to include in these experiments both pepsin and trypsin, in addition to rennet; but, unfortunately, attempts to sterilise pepsin solutions by filtration with the Seitz apparatus were unsuccessful, since the enzyme was retained by the filter. Solutions of rennet and trypsin, however, easily passed through Seitz filters.

The sterilised enzymes were added to autoclaved skim milk and maintained at 20° C. With rennet, six days' incubation was employed; with trypsin one day was sufficient.

Analytical results typical of a number of experiments are presented in Tables I and II below.

It will be observed from Table I that sterilisation did not weaken the rennet significantly, *e.g.* the non-protein nitrogen value (8·0 per cent.) obtained with unsterilised rennet + chloroform, was indistinguishable from the corresponding non-protein nitrogen figure (7·9 per cent.) for sterilised rennet + chloroform. A greater effect was observed with trypsin, *e.g.* a value of 33·2 per cent. for non-protein nitrogen with the unsterilised enzyme + chloroform was reduced to 25·9 per cent. after the trypsin had been sterilised.

The activity of rennet was reduced by 1·6 per cent. chloroform to about one-half. Van Slyke and co-workers did not take into account this consider-

able weakening of rennet by chloroform, and therefore erroneously attributed a very limited role to this enzyme in cheese-ripening.

Table I. *Restraining effect of toluene and chloroform upon proteolysis induced in milk by rennet and trypsin. Results expressed as percentages of the total nitrogen.*

	Rennet, 2 %				
	Sterilised rennet	Sterilised rennet +1·6 % toluene	Sterilised rennet +1·6 % $CHCl_3$	Unsterilised rennet +1·6 % toluene	Unsterilised rennet +1·6 % $CHCl_3$
Non-protein N	14·0	11·9	7·9	12·5	8·0
Proteose N	11·0	9·0	5·5	9·3	5·5
Peptone N	1·4	1·2	0·8	1·4	1·0
Subpeptone N	1·6	1·7	1·6	1·8	1·5
	B.D.H. trypsin, 0·1 %				
	Sterilised trypsin	Sterilised trypsin +1·6 % toluene	Sterilised trypsin +1·6 % $CHCl_3$	Unsterilised trypsin +1·6 % toluene	Unsterilised trypsin +1·6 % $CHCl_3$
Non-protein N	30·8	27·4	25·9	41·4	33·2
Proteose N	18·1	15·1	14·3	21·2	16·2
Peptone N	7·8	7·4	7·0	12·2	9·0
Subpeptone N	4·9	4·9	4·6	8·0	8·0

Table II. *The effect of toluene and chloroform upon the type of proteolysis induced in milk by rennet and trypsin. Results expressed as percentages of the non-protein nitrogen.*

	Rennet, 2 %				
	Sterilised rennet	Sterilised rennet +1·6 % toluene	Sterilised rennet +1·6 % $CHCl_3$	Unsterilised rennet +1·6 % toluene	Unsterilised rennet +1·6 % $CHCl_3$
Proteose N	78·2	75·7	70·0	74·2	69·3
Peptone N	10·3	10·3	10·5	11·3	12·1
Subpeptone N	11·5	14·0	19·5	14·5	18·6
	B.D.H. trypsin, 0·1 %				
	Sterilised trypsin	Sterilised trypsin +1·6 % toluene	Sterilised trypsin + 1·6% $CHCl_3$	Unsterilised trypsin +1·6 % toluene	Unsterilised trypsin +1·6 % $CHCl_3$
Proteose N	58·9	55·1	55·2	51·2	48·7
Peptone N	25·4	27·1	26·9	29·5	27·2
Subpeptone N	15·7	17·8	17·9	19·3	24·1

With rennet, Table II illustrates that there was also a definite small change in the type of breakdown induced by the use of antiseptics, the variation being in the direction of more complete degradation (*i.e.* a higher proportion of subpeptone and less proteose) and assuming more prominence when chloroform was employed.

II. PROTEIN DEGRADATION IN CHLOROFORMED CHEESE.

Series 1. *The effect of chloroform upon the type of breakdown occurring in cheese.*

A number of samples of normal Cheddar cheese, varying in age from 7 to 18 days, were finely ground, and weighed quantities were mixed with excess chloroform (50 per cent. by weight). After storage for periods of 14 days to 3 months at the same temperature as the normal control cheeses, in closed glass jars, portions from both sets of cheeses were analysed. Bacteriological examination of the chloroformed cheeses, both before and after removing the chloroform under diminished pressure, failed to reveal the presence of any organisms at periods after a fortnight.

Typical analytical figures are given in Table III.

Table III. *The effect of chloroform upon the type of protein degradation occurring in cheese.*

	Cheese 1	Control	Cheese 2	Control	Cheese 3	Control	Cheese 4	Control
Age when chloroformed	7 days	—	7 days	—	7 days	—	18 days	—
Period of ripening in presence of $CHCl_3$	14 days	—	1½ months	—	3 months	—	1½ months	—
Percentage of soluble N (expressed as % total N)	10·2	15·1	11·6	18·9	12·7	23·4	18·1	27·0
Expressed as % non-protein N:								
Proteose N	34·0	32·2	23·9	26·0	13·7	17·5	22·6	20·2
Peptone N	41·9	39·6	47·0	40·1	54·8	38·5	50·9	44·6
Subpeptone N	24·1	28·2	29·1	33·9	31·5	44·0	26·5	35·2

It is evident that while the mode of protein decomposition under the influence of chloroform was similar on the whole to that occurring in the control cheeses, yet there was a definite tendency for less complete degradation in the chloroformed cheeses, the subpeptone being lower and the peptone higher in comparison with the values for the control cheeses. This change is the more significant because, in the experiments upon milk in the preceding section, the addition of chloroform resulted in the rennet bringing about a relative *increase* in the proportion of subpeptone. It is realised the objection may be raised that the states of the two sets of cheese were dissimilar, the chloroformed cheeses being in a finely ground condition—but, since bacterial action was excluded in the latter cheeses, it does not seem likely that this difference of state was important.

Series 2. *The effect of adding chloroform at the salting stage.*

Milk of good quality was flash-pasteurised at 155° F. and, equal amounts of the same starter being used in two vats, normal cheese-making procedure was employed up to the salting stage. The first vat was set with the normal amount of rennet, while in the second vat twice this quantity was employed.

The curd (20 lb.) from vat 2, after salting, was divided into two equal portions, one of which served as a control. The other portion was finely minced

and mixed with chloroform (2 lb. = 20 per cent.). Both lots of curd were hooped and pressed.

The curd (20 lb.) from vat 1 was also divided equally after salting and an additional quantity of rennet (8 drams, equivalent to nearly three times the proportion employed in setting) was dissolved in water (200 ml.) and incorporated in one portion which was chloroformed and hooped as above, the other half of the curd being pressed into a control cheese. All the cheeses were stored at the same temperature, the chloroformed ones (which lost a small amount of fat during pressing) being kept in desiccators in an atmosphere saturated with chloroform.

Samples from the cheeses were taken at intervals for bacteriological examination and analysis. To conserve space the results are depicted graphically. In Figs. 1 and 2 the nitrogen values are expressed as percentages of the total nitrogen, whereas Figs. 3 and 4 show the partition as percentages of the non-protein nitrogen. Logarithms of bacterial counts are plotted.

From Figs. 1 and 2 it is evident that the general course of protein breakdown was similar for all the cheeses. In Figs. 3 and 4 the curves display, at an early stage, definite divergence for the two sets of cheeses, the proportion of subpeptone being greater in the control cheeses, while the other constituents were correspondingly smaller. After about 5 weeks the curves for any given breakdown constituent in the chloroformed and normal cheeses tend to remain almost parallel. The interpretation of these curves will be discussed at greater length under series 3.

It should be mentioned that, owing to the rubbery nature of the curd when chloroformed, complete mixing was not obtained, and as a result the cheeses were at no time completely sterile. It is considered unlikely, however, that such small numbers of bacteria as remained would influence the ripening process.

Series 3. *The effect of adding chloroform* (*and additional rennet*) *in the early stages of ripening.*

(*a*) Weighed portions (400 g.) of a normal cheese were, at two different early stages in its ripening (18 and 40 days respectively) mixed with rennet (1·5 ml. = 0·375 per cent.), equivalent to 1·5 times the proportion employed for setting) dissolved in water (20 ml.). Chloroform (200 g. = 50 per cent.) was incorporated, and plate counts and analyses were carried out at several later dates. The cheeses were invariably found to be sterile a fortnight after the addition of chloroform.

The results of analyses at intervals are given in Table IV.

(*b*) One-kilo portions from two normal cheeses (1 week old) manufactured according to standard procedure, were finely ground, rennet (3·75 ml. = 0·375 per cent., equivalent to 1·5 times the proportion employed for setting) dissolved in water (70 ml.) incorporated, and chloroform (350 ml.) well mixed in. Bacteriological examination at intervals revealed that the cheeses remained

Table IV. *The nitrogen changes in cheese in the presence of chloroform (and additional rennet) added in the early stages of ripening.*

	Cheese 1	Control	Cheese 1	Control	Cheese 1	Control	Cheese 2	Control	Cheese 2	Control
Age when chloroformed	18 days	—	18 days	—	18 days	—	40 days	—	40 days	—
Period of ripening in presence of $CHCl_3$	3 weeks	—	7 weeks	—	11 weeks	—	4 weeks	—	8 weeks	—
Nitrogen as % total N:										
Soluble N	25·3	23·9	28·7	28·3	30·4	31·8	28·7	28·3	31·4	31·8
Non-protein N	21·7	19·0	24·5	24·2	26·3	26·5	25·5	24·2	27·6	26·5
Proteose N	6·7	4·7	6·7	4·9	6·0	3·2	6·3	4·9	5·0	3·2
Peptone N	10·1	8·3	11·8	10·8	12·4	11·8	11·7	10·8	13·6	11·8
Subpeptone N	4·9	6·0	6·0	8·5	7·8	11·5	7·4	8·5	9·0	11·5
Nitrogen as % of non-protein N:										
Proteose N	30·9	24·7	27·5	20·2	22·9	12·1	24·9	20·2	18·0	12·1
Peptone N	46·4	43·9	48·1	44·6	47·2	44·6	46·1	44·6	49·3	44·6
Subpeptone N	22·7	31·4	24·4	35·2	29·9	43·3	29·0	35·2	32·7	43·3

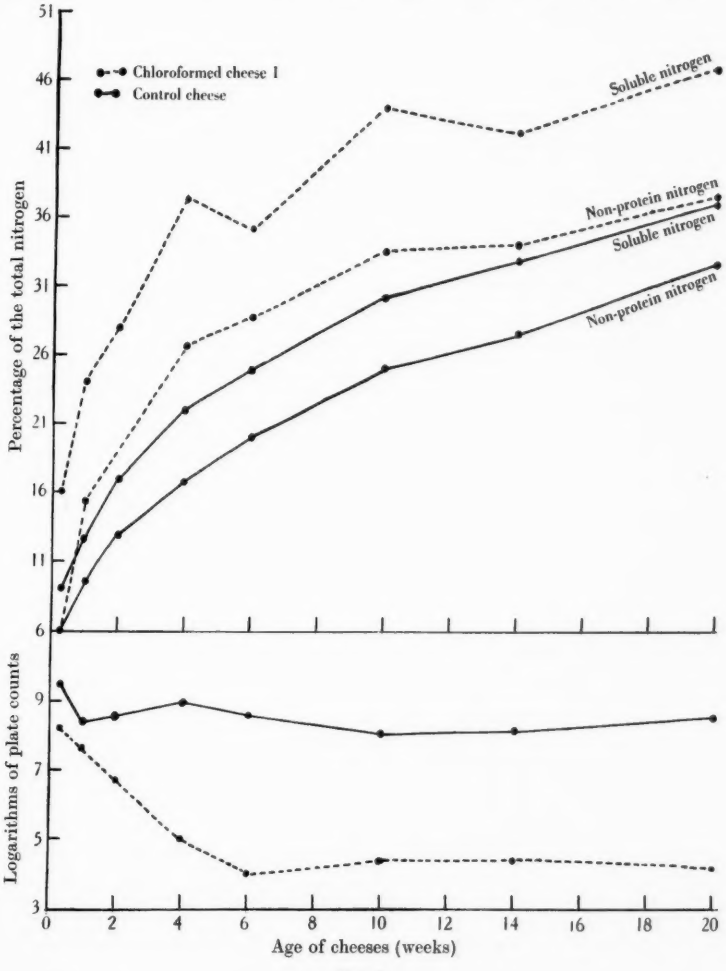

Fig. 1.

sterile. The progress of ripening in both these cheeses and their controls was followed over an interval of 19 weeks.

The results are shown in Figs. 5 and 6. To avoid confusion, the curves for subpeptone nitrogen have been omitted from Fig. 6. These curves (which

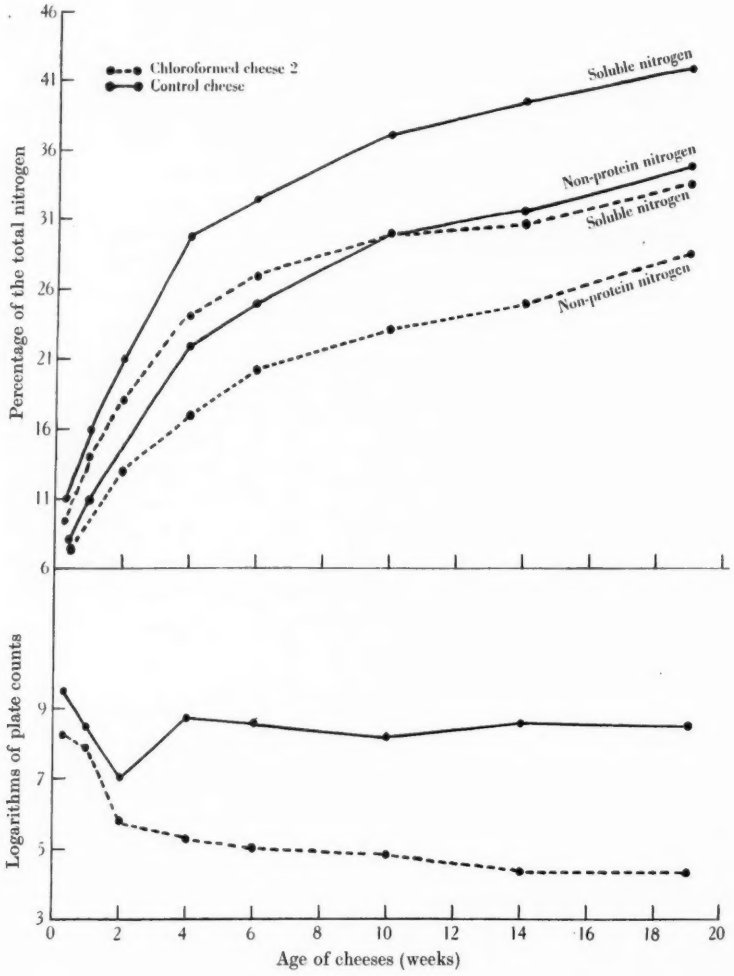

Fig. 2.

display essentially the same features as the corresponding curves in Figs. 3 and 4) may be reconstructed by subtracting proteose nitrogen + peptone nitrogen from the corresponding non-protein nitrogen values.

Discussion of the three series.

The cheeses of series 2 contained amounts of rennet which yielded breakdown in the resulting chloroformed cheeses, in one instance less and in the other greater than that occurring in the corresponding control cheeses. In series 3 the proportion of added rennet was adjusted so as to yield the same amount of protein degradation.

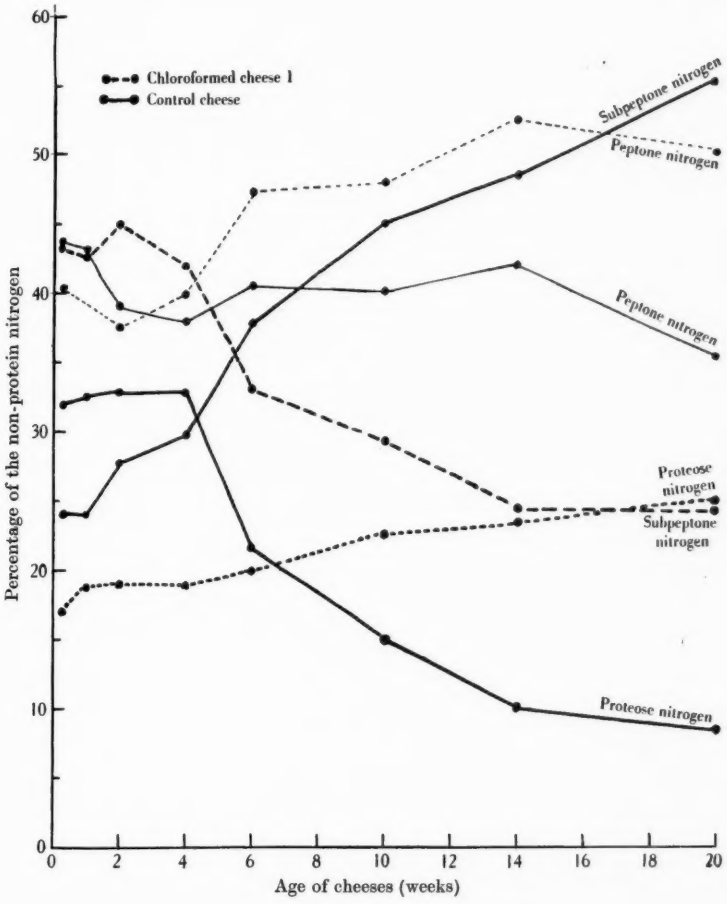

Fig. 3.

In none of these experiments was the general course of breakdown, as judged by determination of soluble nitrogen and non-protein nitrogen, dissimilar from that obtaining in the corresponding control cheeses, and indeed, in series 3, Fig. 5 reveals identical protein attack throughout the ripening period. If micro-organisms or galactase played any important role in degrading

protein, it appears unlikely that this absolute correspondence for normal and chloroformed cheeses during ripening, evidenced by Fig. 5, would have been obtained.

In the majority of the cheeses treated with chloroform it was necessary to dilute the added rennet with water before mixing with the ground cheese, in

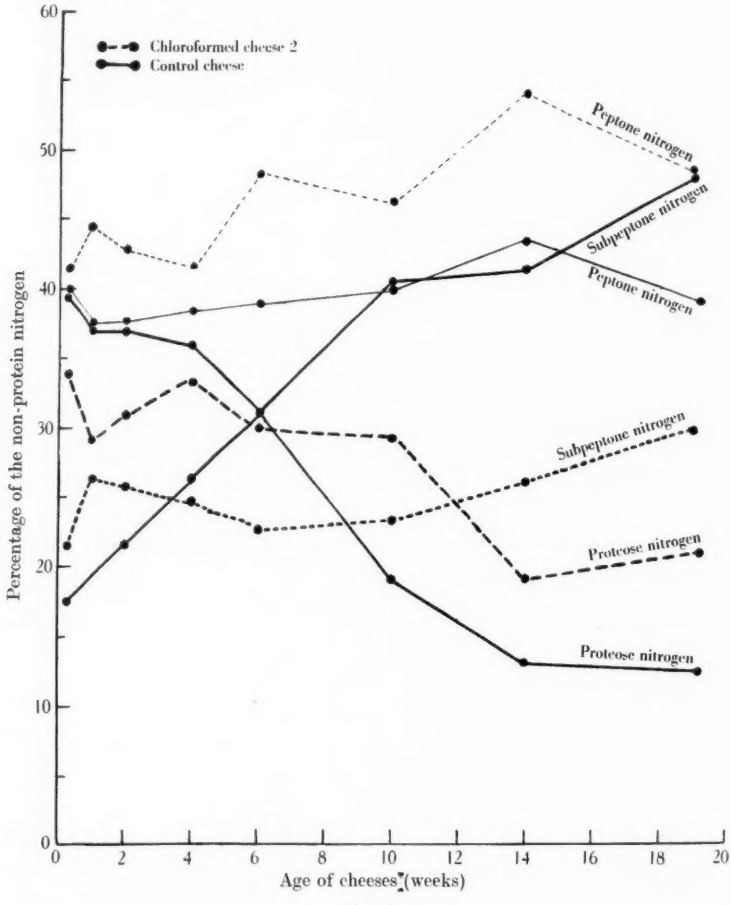

Fig. 4.

order that uniform incorporation might be obtained. It is possible that objection may be raised to the higher moisture content of these cheeses, since it is well known that in normal cheese a high moisture content leads to greater protein breakdown. This fact, however, has no bearing upon the present work, since in the chloroformed cheeses employed it was only enzyme action that functioned; so that the effect of additional water would be expected to be

merely one of dilution, resulting in all the stages of breakdown being proportionally retarded to a small extent. The validity of this assumption received support from the fact that cheese 2 in series 2, to which no water had been added, behaved in exactly the same manner during ripening as the remainder of the cheeses in series 2 and 3 which contained added water.

The essence of the results obtained here may be expressed thus: The incorporation of chloroform (which also led to the destruction of the bacterial

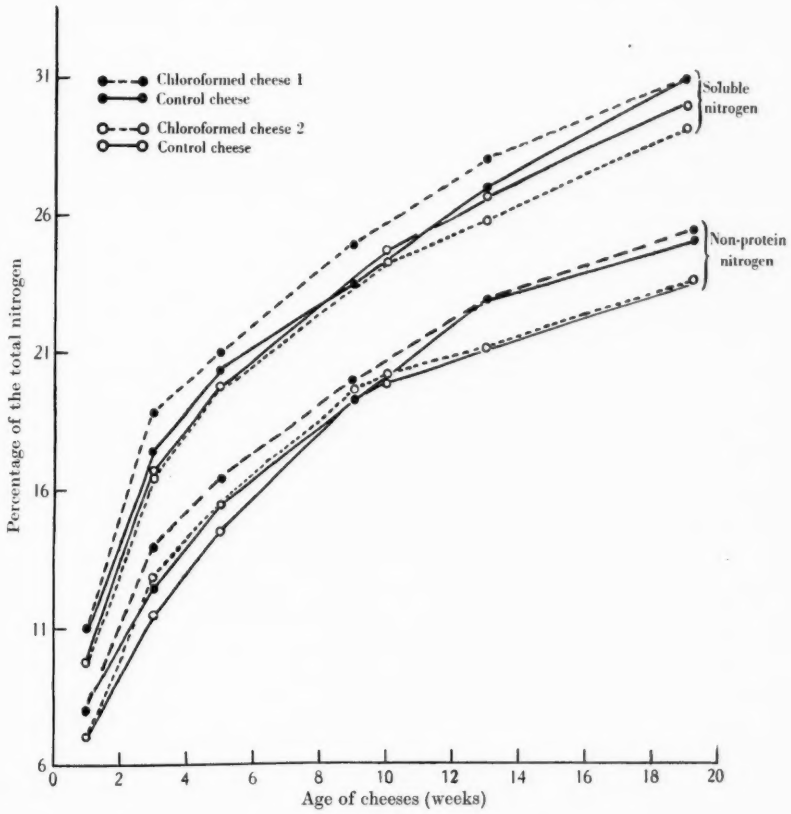

Fig. 5.

flora) was found to reduce markedly the extent of protein breakdown occurring in cheese, but by the addition of rennet, the diminution in breakdown was completely restored, so that the curves depicting soluble nitrogen and non-protein nitrogen for the chloroformed material coincided throughout ripening with the corresponding curves obtained for the normal control cheeses. It is felt that these experiments afford strong evidence for the paramount role of rennet in attacking cheese protein during ripening. It is also apparent that

the very large decrease in the extent of breakdown occurring in chloroformed cheese, observed by Van Slyke and his co-workers, was not due to the presence of some other active agent in the normal cheese, but was simply caused by weakening of the rennet through the presence of chloroform.

A consideration of the partition of nitrogen between the non-protein constituents (Figs. 3, 4 and 6) reveals two salient features:

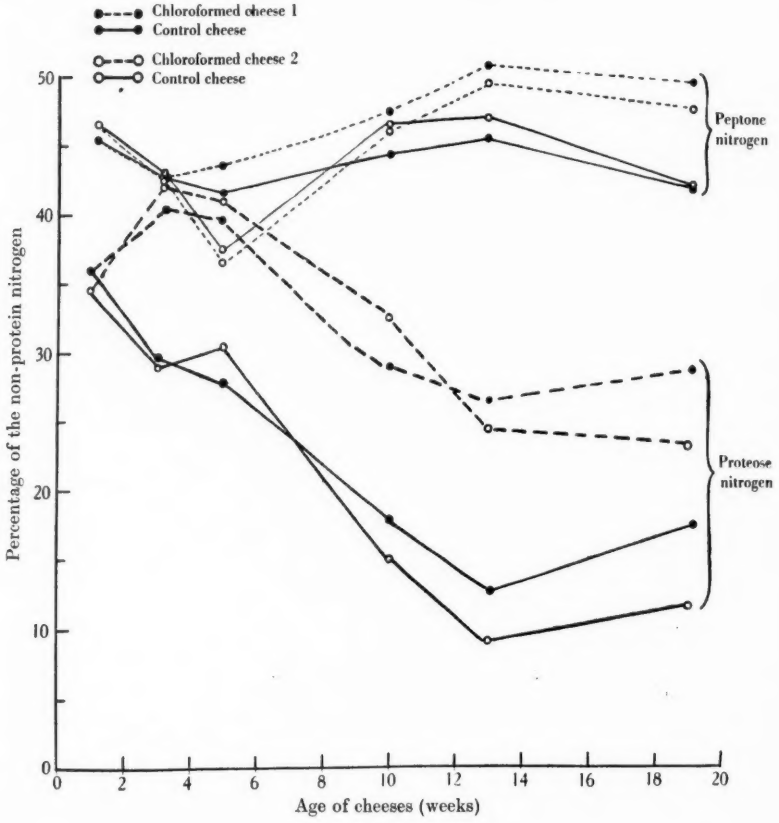

Fig. 6.

(1) In all the chloroformed cheeses examined the proportion of subpeptone was invariably lower than in normal cheese.

(2) The curves for each of the non-protein constituents of the chloroformed and corresponding normal control cheese run approximately parallel over the greater part of the ripening period.

The first finding is in agreement, qualitatively, with the results of Van Slyke and his collaborators, but, whereas they, working with cheese abnormally low in acid, obtained for chloroformed and normal cheese an average ratio of

amino acid values equal to 1:9, the experiments described here yielded a maximum ratio of 1:2 for subpeptone nitrogen. The method for estimation of nitrogen partition described by Van Slyke and co-workers is essentially similar to that employed in this investigation, and there is no reason for assuming that the two methods would not yield comparable results. The consistently lower proportion of subpeptone nitrogen in chloroformed cheese appears to be capable of explanation in three ways:

(*a*) Streptococcal enzymes, after death of the organisms, may conceivably exert a continued influence upon the non-protein nitrogen distribution. This does not seem likely to be a factor of much importance, however, since the cocci usually disappear very early in cheese-ripening.

(*b*) Galactase, which would undoubtedly be considerably restrained in its action by chloroform (8) may be partially responsible for the larger proportion of subpeptone in normal cheese.

(*c*) Lactobacilli, while unimportant in the destruction of protein during ripening, may yet be capable of resolving proteose (and peptone) still further to subpeptone. Experiments by the author have shown definitely that, in digested milk, many strains of lactobacilli are capable of breaking down large amounts of proteose, and investigations with sterilised cheese as a medium indicate that a similar action also occurs in this case.

Completion of these experiments will, it is hoped, yield more definite information upon these points.

In regard to the second point of significance emerging from a study of Figs. 3, 4 and 6, viz. the parallel courses of each non-protein constituent in the normal and chloroformed cheeses during ripening, it is clear that even in cheese where bacterial action has been eliminated, the relative proportion of proteose continues to diminish and the subpeptone rises during ripening in the same manner as in normal cheese, although, of course, the *actual amounts* of these constituents present at a given time are not the same in chloroformed cheese as in normal cheese. It is possible that this progressive change in partition may result from prolonged rennet action, the proteose accumulating during the ripening process gradually undergoing further attack. It is worthy of notice, in this connection, that whereas rennet action in milk appears to cease after a short interval (about 2 weeks at 20° C.) yet in cheese the enzyme continues to break down protein over very long periods. Other possible factors in this non-protein partition "drift", which have to be borne in mind, are the presence of weakened galactose and the survival of weakened bacterial enzymes.

III. Experiments on the development of flavour in cheese ripened in the presence of chloroform.

The cheeses considered in the sections above were all examined for the development of flavour.

Methods employed. 100 g. of the cheese (containing chloroform) was

ground, if necessary, and exposed to the atmosphere for about an hour, with occasional stirring. At the end of that time most of the chloroform had disappeared, and the sample was then steam-distilled. The first portion of the aqueous distillate (containing chloroform) was rejected. The characteristic Cheddar aroma, if present, could easily be detected in the distillate following. Control distillations on normal cheese were always carried out simultaneously for purposes of comparison. After the volatile material had ceased to distil, the presence or absence of a very marked and characteristic aroma in the residual curd afforded confirmation of the possession or lack of flavour in the original material.

The method was checked in a large number of experiments upon mature cheese and young cheese in which flavour had not yet appeared. The incorporation of chloroform with the finely ground cheese before examination did not affect the results obtained. The mature cheese was very easily distinguished in every instance, and the method was so sensitive as to differentiate in the early stages of ripening between cheeses manufactured from pasteurised milk and those made from raw milk (in which flavour develops more rapidly).

Results. All the cheeses described in series 2 and 3 above were examined at intervals. In no case was there any doubt about the absence of Cheddar flavour in the cheeses ripened in the presence of chloroform, which were analogous, in this respect, to very young cheese. Since the volatile acids in cheese are most likely to arise from bacterial action, the volatile acidity of chloroformed cheese would not be expected to increase during ripening, and this was found to be the case in actual practice.

It thus appears that the views commonly held, ascribing the formation of flavour-producing substances to micro-organisms, are correct, no flavour developing through rennet action.

This investigation furnishes further evidence for the correctness of the deduction advanced in a previous communication (7) that there is no necessary direct correlation in cheese between flavour and nitrogen partition (as usually determined). The only possibly significant results yielded by nitrogen partition determinations appear to be that in chloroformed cheese (in which flavour does not develop) the proportion of subpeptone is smaller than in normal cheese, while raw milk cheeses contain a higher percentage of subpeptone than do pasteurised milk cheeses, corresponding to earlier flavour development.

Summary.

Attempts were made to eliminate the bacterial factor in Cheddar cheese-ripening, through the agency of chloroform, and an assessment of the role of rennet was thus rendered possible.

After investigating the effect of chloroform and toluene upon the proteolytic action of rennet and trypsin in milk, the results obtained were applied in studies upon the ripening of cheese in the presence of chloroform. Cheese of normal manufacture was chloroformed at the salting stage or one week or

more after manufacture (when the curd had lost its rubbery nature and the germicide was more easily incorporated). Owing to the weakened activity of enzymes in the presence of germicides, it was necessary to employ larger proportions than usual of rennet, measured additions generally being made at the time when the chloroform was added. Under these conditions the general course of protein degradation, as measured by determinations of soluble nitrogen and non-protein nitrogen, was found to be identical with that occurring in normal control cheeses. Strong evidence was thus afforded that rennet is the only important agent attacking cheese protein during the ripening process.

In the partition between the non-protein constituents of the normal and chloroformed cheeses, respectively, there were observed definite differences which tended to remain constant over the greater part of the ripening period. The relatively higher proportion of subpeptone nitrogen in the normal cheese was shown to be due, at least in some measure, to bacterial action.

Cheese ripened in the presence of chloroform developed no volatile acid, and no cheese flavour could be detected.

The author's thanks for criticism and advice are due to Dr H. R. Whitehead and Dr F. H. McDowall.

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107. STUDIES ON THE CHEMISTRY OF CHEDDAR CHEESE MAKING

I. THE MINERAL CONTENT OF CHEESE AND WHEY

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(With 4 Figures.)

The timing of operations in the manufacture of Cheddar cheese is based partly on observations by the cheese maker of the condition of the curd at the various stages and partly on determinations of the acidity of the whey exuding from the curd. The efficacy of the first means of control is dependent on the experience and judgment of the cheese maker. The usefulness of the second method depends on an accurate knowledge of the significance of the acidity figures obtained.

The acidity determination was introduced by Lloyd in the course of his *Investigations in Cheesemaking*(1) in the south-western districts of England during the years 1892–8, and was introduced into America at about the same time by Van Slyke and his co-workers. It is now regularly used by Cheddar cheese makers throughout the world.

The general practice in New Zealand has been to rely more on the determination of acidity than on the simple judgment of the cheese maker. It has been found, however, that the acidity is not always a reliable indication, especially in the later stages of the process, and there has recently been a tendency to return to the use of the hot iron test to assist in controlling the later operations.

It frequently happens that there is a fall in the whey acidity after the milling stage and especially after the curd is salted, though from the feel and texture of the curd there may be every indication that acid development in the curd is proceeding normally. Such a fall in acidity may be attributed partly to the increased fat content of the later wheys and, with salted wheys, to dilution of the other constituents of the whey by the large percentage of salt present. These factors alone, however, are not sufficient to account for the whole of the fall of acidity noted. In other cases, too, the acidities of the wheys observed during the making process may have no visible relationship to the type of cheese obtained—an "acid cut" cheese may result from a vat of milk in which the progress of acidity development was apparently not very abnormal, and *vice versa*. An incorrect estimate of the moisture content of the curd may contribute to the occurrence of such faults, since the amount of

lactose incorporated in the cheese and hence the potential acidity development will be influenced to a great extent by the quantity of whey in the curd at hooping. The amount of unfermented lactose and of minerals capable of neutralising acid will, however, also depend on the quantities of lactic acid formed earlier in the process, so that an accurate control of acid development is essential to ensure a uniform product.

It seemed to the present authors that an examination of the composition of the whey exuding from the curd at the various stages would possibly offer an explanation of the anomalies mentioned and would enable a more accurate interpretation of acidity readings.

The present paper deals with the first of several investigations which have been carried out along these lines at this Institute. Some phases of the investigation have already been partially covered by the work of Lloyd (1) which unfortunately did not come to the notice of the authors until a late stage in their work. Although Lloyd's figures refer to Cheddar cheese made according to a somewhat different procedure from that now practised in New Zealand, in that starter was not added to the cheese milk in order to control the type of bacterial action and the rate of acid production, his results and conclusions show a remarkably good agreement with those detailed below. Lloyd noted the effect of both heat and acidity in causing the contraction of the curd and expulsion of whey, the fact that acid production goes on in the curd and the existence of a relationship between acidity production and the amount of whey in the curd. He used the acidimetric titration of the whey to follow through the course of the cheese-making process and devised rules for its use in timing the operations. He also carried out analyses on the wheys obtained at various stages and noted the weight of whey lost at each stage. He showed that there was a steady rise in the concentration of total ash and of calcium in the successive wheys throughout the process.

Taking into account a number of examples, Lloyd concluded that "the higher the percentage of lactic acid developed in the curd, the smaller the proportion of lime which is left in that curd", and that the lime in the curd must be combined with the casein. In summarising his results he states that "of the total solids present in the milk, one-half is lost in cheese making. The curd retains the whole of the casein, most of the fat, about one-third of the mineral matter and a very small quantity of sugar. The remainder of the sugar and mineral matter, as also the albumen, pass off in the whey and other drainings from the curd."

Sammis, Laabs and Suzuki (2) showed that the acidity within the curd cubes after cutting was always greater than that in the whey outside the curd. Their method was, however, open to some objections, since they used a considerable volume of water in extracting the acid from the curd, and in all probability their solutions contained a proportion of buffer substances dissolved from the curd. Their results are therefore not a reliable indication of the actual amount of lactic acid formed within the curd.

Since this early work few investigations have been made on the composition of wheys obtained in cheese making. Recently Wode (3) has shown that the quantity of calcium dissolved from the curd increases with the acidity of the milk before setting and the quick separation of vat whey means a smaller loss of calcium. Wode's original paper has, unfortunately, not been available to the present authors and the quotation is from an abstract (4).

Some light has been shed on the reactions involved in cheese making by work done on the changes brought about in milk by souring. Van Slyke and his co-worker (5) showed that with the development of lactic acid the insoluble dicalcium phosphate of the milk is converted into soluble monocalcium phosphate, while the insoluble calcium caseinates are changed into soluble calcium lactate and caseinates containing less calcium, and finally into uncombined casein.

Grimmer and his co-worker (6), in studies on the souring of milk, determined nitrogen, calcium and phosphorus in the ultrafiltrate of milk and in the whey from rennet curd at various degrees of souring. At low acidities the rennet whey was found to contain less calcium and phosphorus than the ultrafiltrate of milk. It was therefore suggested that soluble calcium and phosphorus compounds in the milk were removed from solution during the rennet coagulation either by being entangled in the curd or by the formation of a complex of calcium paracaseinate and calcium phosphate. With souring little relative change in the proportions of nitrogen to calcium to phosphorus in the rennet curd were found at acidities below 0·18 per cent. (calculated as lactic acid), indicating that the calcium-phosphorus-casein complex was unaltered at this acidity and the lactic acid formed must be neutralised by other substances in the milk. With further increase in acidity there was a regular rise in the calcium content of the whey, one atom of calcium per 100 atoms of curd nitrogen passing into the whey for each 0·045 per cent. rise in acidity. The rise in phosphorus content was much slower and less regular. The reason given was that the action of lactic acid on calcium caseinate and tricalcium phosphate would bring only calcium into solution, although its action on dicalcium phosphate would liberate equal numbers of atoms of calcium and phosphorus as soluble compounds.

A number of investigations have been made of the mineral content of cheese, but in only a few cases have the results been correlated with the details of manufacture.

Nottbohm and Baumann (7) give figures showing that the mineral contents of various types of cheese depend on the method of manufacture and chiefly on the acidity developed. Hard rennet cheeses have 5·1–8·7 per cent. of salt-free ash in the fat-free dry matter (Canadian Cheddar 6·16 per cent.), while cheeses depending partly or wholly on the development of acid for coagulation of the milk have ash contents ranging as low as 1 per cent. in the case of sour milk cheeses.

McCann, Caulfield and Kramer (8) give analyses of various types of cheese

made under controlled conditions. A number of the details of manufacture are given but no figures of the acidities developed are recorded. These authors state that "the phosphorus is proportional to the protein in cheese made from clotted casein, regardless of the methods of manufacture, even though they involve different chemical reactions". Their figures show a range of 0·010 g. phosphorus per gram protein for acid cottage cheese to 0·016 for Neuchatel (American Cheddar 0·011). Assuming that casein contains 0·85 per cent. phosphorus, they calculate that some 85 per cent. of the phosphorus in Cheddar cheese is accounted for by the phosphorus content of the casein. Calculation from cheese yields shows that 30–44 per cent. of the phosphorus of the milk is retained in the cheese (Cheddar 38·1 per cent.), while the figure for calcium varies from 13 to 22 per cent. in Neuchatel and 17–25 per cent. in acid cottage cheese to 80·4 per cent. in Cheddar.

Methods.

Samples of whey were collected from a vat in which cheese was being made from a large volume of milk (either 800 or 3000 lb.). After filtration through filter-paper or glass-wool (samples of whey collected later in the process than 'drying"[1] do not filter readily through filter-paper), the wheys were analysed for total solids, calcium, phosphorus, chlorine, total nitrogen and, in a few instances, for citric acid and sodium + potassium. No attempt was made to separate sodium and potassium, as it was considered the figures for the individual elements would have no practical significance. Densities of all wheys were determined, to allow calculation of the results on a weight basis.

Total solids. 4 ml. of the sample were weighed in a metal dish and dried in a steam oven for 5 hours or to constant weight.

Density. The weight delivered by a 4 ml. pipette was determined in weighing out the sample for the total solids determination. The density thus determined was found to agree with that obtained using 10 or 20 ml. pipettes employed for measuring out samples for other determinations.

Calcium. The milk or whey (25 ml.) was evaporated to dryness after addition of a few drops of dilute acetic acid and the residue was ashed at a low red heat. The calcium was determined volumetrically by titration of the oxalate precipitated in slightly acid solution at the boiling-point, the solution when cold being made neutral to methyl red with sodium acetate and then allowed to stand. In a few cases calcium was determined directly on the filtrate obtained by precipitation of the proteins with trichloracetic acid.

Phosphorus. 5 or 10 ml. of the sample were digested with concentrated nitric and sulphuric acids, and the phosphate was estimated gravimetrically as phosphomolybdate, after drying at 180° C. The values for phosphorus given in this paper are total phosphorus. No attempt has been made to distinguish between organic and inorganic phosphorus.

[1] The "drying" stage, referred to in this paper, is the New Zealand equivalent to "piling" in British cheese-making procedure.

Chlorine. The estimation was carried out on a volume of 20 ml. of whey (except for the salter wheys which were diluted and an aliquot taken) by the method described by McDowall and Whelan (9) for cheese, with the modification that the precipitated silver chloride was filtered off before titration.

Sodium + potassium was estimated by the method of Brown and Shohl (10).

Citric acid. Protein was removed by the procedure of Supplee and Bellis (11), and the citric acid determined gravimetrically as pentabromacetone by the A.O.A.C. method (12).

Degree of uniformity in whey composition.

On four separate occasions milk was made into cheese, under as nearly as possible identical conditions, in two separate 10-gallon vats. Determinations were made of total nitrogen, fat, calcium and phosphorus in the wheys, and of moisture and salt in the cheese. The greatest differences found between the two vats barely exceeded the experimental error of the analyses.

Table I. *The relation of titratable acidity to the calcium and phosphorus contents of wheys.*

Raw milk used. Analysis: total solids 13·22 %; fat 3·71 %; total nitrogen 0·574 %; casein 2·78 %; CaO 0·172 %; P_2O_5 (total) 0·255 %; starter 1 %; rennet 4 oz./1000 lb.

Description of sample	Time after cutting (min.)	Acidity (g. lactic acid)/100 ml.	CaO % g./100 ml.	P_2O_5 (total %) g./100 ml.
Cutting	10	—	0·052	0·128
	85	0·165	0·060	0·131
	125	0·180	0·064	0·135
Running	145	0·185	0·063	0·137
Last stage of running	155	0·215	0·080	0·144
Drying	157	0·245	0·088	0·154
	159	0·26	0·120	0·159
	170	0·31	0·122	0·172
	180–182	0·36	0·157	0·185
	192–196	0·425	0·196	0·206
	220–225	0·56	0·294	0·255
	225–237	0·61	0·323	0·267
Milling to salting	335	0·95	0·525	0·381

Variations in the mineral composition of wheys.

Estimations were made on whey samples taken at intervals throughout the process on a number of different days of normal cheese making. A typical set of results is set out in Table I and graphically in Fig. 1. The concentrations of calcium and phosphorus and the titratable acidity in the whey rise very slowly to the running stage, but from then on to salting there is a rapid increase in all three.

In the second series of analyses on wheys from one vat, particular attention was paid to wheys obtained after salting. Determinations were made of total ash, soluble ash and insoluble ash and of calcium and phosphorus in both portions of the ash as well as directly on the wheys (see Table II).

There is a rise in total ash throughout the process. The percentage of soluble ash in the total ash is much higher in the first wheys (57, 48, 41 per cent.) than in the milk (36 per cent.), on account of the greatly decreased amounts of calcium and phosphorus in the early wheys as compared with the milk. With the later wheys the percentage of soluble ash in the total ash falls, due to increasing concentrations of calcium and phosphorus in the whey. When between drying and milling the concentration of calcium and phosphorus in the whey exceeds that in the original milk, the percentage of soluble ash in the total ash of the whey (34 per cent.) becomes less than the corresponding figure for milk.

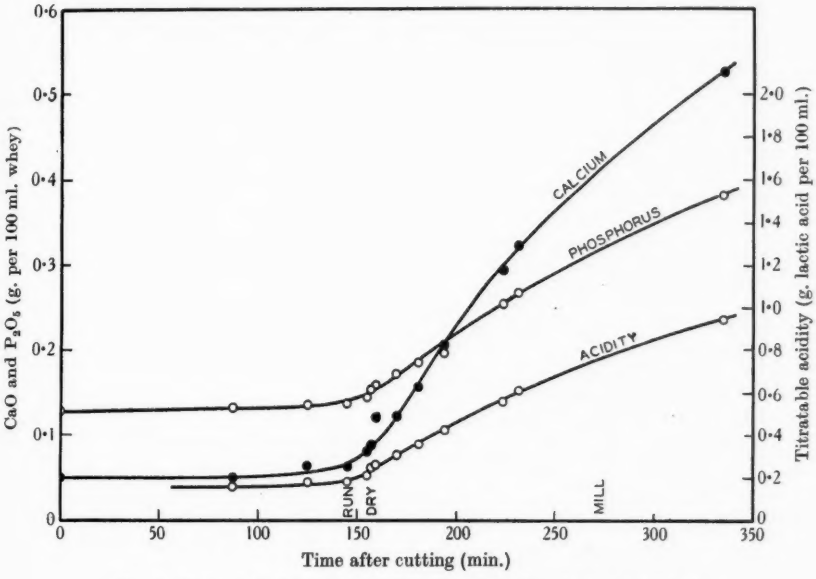

Fig. 1. Changes in the acidity and calcium and phosphorus content of wheys during the cheese-making process.

In the ash of the milk about 10 per cent. of the total phosphorus and 3 per cent. of the total calcium are present in the soluble ash. In the first whey the figures are higher (P 20 per cent.; Ca 5 per cent.). The amounts of soluble calcium and phosphorus in the ash decrease as the process continues till just before salting less than 1 per cent. of the total phosphorus and less than 0·5 per cent. of the total calcium is present in the soluble ash. After salting there is a temporary rise in the soluble phosphorus to 3–4 per cent. of the total followed by a fall in the last salted wheys and the press whey. The calcium of the salted wheys is almost entirely present in the insoluble ash. The rise in phosphorus in the soluble ash after salting seems likely to be due to the solution of some of the phosphate of the ash as sodium phosphate.

Table II. *Composition of Cheddar cheese whey, more particularly the whey after salting.*

Raw milk used. Analysis: fat (Babcock) 4·0 %; total nitrogen 0·538 %; casein (Walker) 2·53 %; quantity of milk 3127 lb.; starter 1 %; rennet 4 oz./1000 lb.

Sample No.	Description of sample	Time after cutting (min.)	Acidity (g. lactic acid)/100 ml.	Total solids g./100 g.	Ash g./100 ml.			Cl direct g./100 ml.	CaO g./100 ml.			P_2O_5 g./100 ml.			CaO/H_2O g./100 g. water	P_2O_5/H_2O g./100 g. water
					Insoluble %	Total %	Ash less salt %		In soluble ash	Total in ash	Direct	In soluble ash	Total in ash	Direct		
1	Milk	—	0·17	13·09	0·521	0·817	—	0·086	0·006	0·178	0·171	0·0232	0·225	0·238	0·191	0·266
2	Whey at running	147	0·16	6·99	0·243	0·566	—	0·102	0·004	0·075	0·074	0·0232	0·115	0·123	0·077	0·128
3	Whey at drying	167	0·24	7·06	0·335	0·645	—	0·092	0·004	0·106	0·103	0·0111	0·136	0·140	0·108	0·146
4	Whey at drying	197	—	6·96	0·478	0·814	—	0·092	0·004	0·189	0·178	0·0030	0·174	0·181	0·186	0·189
5	Whey 15 min. before milling	272	0·81*	9·45	—	—	—	0·099	—	—	0·463	—	—	—	0·497	—
6	Whey just before salting	345	0·98	13·58	1·358	2·052	—	—	0·004	0·505	0·609	0·0036	0·407	0·424	0·682	0·475
7	Whey 0–10 min. after salting	347–357	—	25·49	1·052	20·41	1·79	11·30	0·001	0·303	0·290	0·0136	0·311	0·319	0·335	0·368
8	Whey 10–13 min. after salting	357–360	—	23·67	0·992	19·40	1·44	10·92	0·001	0·291	0·290	0·0103	0·302	0·318	0·328	0·360
9	Whey 13–16 min. after salting	360–363	—	22·22	1·14	18·06	1·66	9·96	0·001	0·296	0·283	0·0088	0·311	0·329	0·317	0·369
10	Whey 16–21 min. after salting	363–368	—	22·44	1·03	16·85	1·63	8·21	0·001	0·317	0·302	0·0131	0·325	0·344	0·343	0·390
11	Whey 21–26 min. after salting	368–373	—	20·35	1·09	14·58	1·69	7·83	0·001	0·344	0·336	0·0107	0·354	0·378	0·376	0·423
12	Whey 26–35 min. after salting	373–382	—	18·79	1·18	12·15	1·43	6·49	0·001	0·356	0·353	0·0065	0·388	0·408	0·394	0·455
13	First 15 min. press whey	400	—	14·11	1·20	6·59	1·23	3·25	0·003	0·401	0·406	0·0033	0·388	0·420	0·444	0·459

* At milling.

The total calcium and total phosphorus in the wheys show a steady rise up to the salting stage in agreement with the results given in Table I. Immediately after salting there is an abrupt fall, which is particularly noticeable for calcium, followed by a fairly rapid rise. Before the values for the composition of the salted wheys can be compared with those of the earlier wheys it is, however, necessary to make allowance for the effect of salt and fat contained therein in diluting the other constituents. The results have therefore been recalculated to a basis of percentages in the water contained in the whey, values for which were derived from the total solids estimations. The adjusted

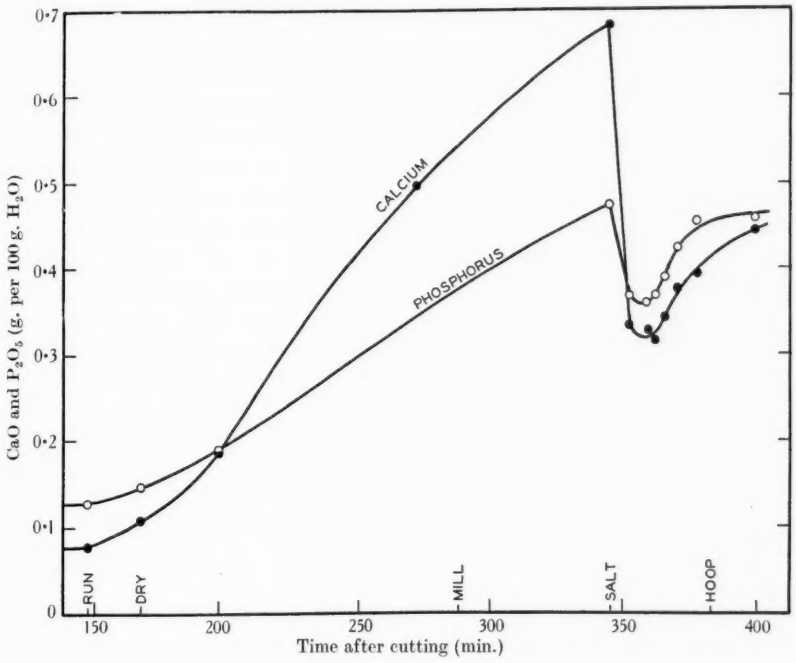

Fig. 2. Changes in calcium and phosphorus content of wheys.

figures are shown on the right-hand columns of Table II from which Fig. 2 has been drawn. It will be seen that the drop in calcium and phosphorus after salting is still evident.

The drop in phosphorus concentration after salting is much less marked than that for calcium, so that the ratio of calcium to phosphorus in the whey is greatly altered after salting.

The most likely explanation of this concentration drop after salting is that it is an osmotic effect, the high concentration of salt added causing an outward diffusion of water from the curd, thus diluting the whey. An alternative explanation is that the dilution is due to a liberation of bound water from the

curd by the dehydrating action of the salt. In any case is seems likely that a Donnan equilibrium effect will also be present and will control the distribution of the ions of the mineral salts between the curd and the whey. The suggestion that the salt releases water from the curd either by osmotic or dehydrating action receives support from the observation that the rate of flow of whey from the curd, which falls almost to zero after milling, is suddenly increased after salting when there is a rapid flow of whey. Further aspects of this phenomenon will be considered in a later paper.

The variations in calcium and phosphorus concentrations noted here were confirmed in later experiments. The chloride content of the wheys up to salting exhibited no significant variations.

Total weights of calcium and phosphorus escaping in whey.

A further experiment was carried out to determine the actual weights of calcium and phosphorus lost at each stage of the cheese-making process. The whole of the whey which came off was collected in a number of portions, each of which was weighed and analysed. The curd was also weighed and sampled for analysis at intervals. It was thus possible to calculate the actual weights of CaO and P_2O_5 present in each portion of whey, and as an additional check the determinations on curd enabled the calculation of an approximate balance at each stage. The results of the analytical determinations are shown in Table III and the actual weights lost at each stage are given in Table IV. Allowance was made for the weights of curd removed in sampling and for the weight of salt added.

At milling there was an unaccounted loss of 21 lb. in total weight, and after 1 day this loss had increased to 25 lb. The greater part of this loss was water, most of which probably escaped by evaporation. There is quite a satisfactory agreement between the weights of CaO and P_2O_5 found present in the curd at each stage and the weights calculated by subtracting the weight lost in the whey from that in the milk.

The figures show that, in this experiment, 40 per cent. of the total calcium in the milk and 43 per cent. of the total phosphorus escaped into the whey. 86 per cent. of the calcium lost and 90 per cent. of the phosphorus lost was found in the whey at running. It will thus be seen that the acidity developed in the early stages of the process, by controlling the quantities of minerals passing into the whey before running, will have a much more important influence on the calcium and phosphorus content of the cheese, than the acidity developed later in the process.

Effect of milk acidity on the calcium and phosphorus lost in the whey.

Preliminary experiments were carried out to determine whether the addition of acid to milk affected the titratable acidity of the milk in a quantitative manner.

Table III. *Composition of wheys and curd in experiment on quantitative distribution of mineral constituents between curd and whey.*

Raw milk used. Analysis: fat (Babcock) 4·2 %; casein (Walker) 2·97 %; casein (Kjeldahl) 2·99 %; starter 1·5 %; rennet 4 oz./1000 lb.

			Whey								Curd				
Sample No.	Description	Time after cutting (min.)	Wt. of milk or whey (lb.)	Acidity (g. lactic acid)/100 ml.	Total solids g./100 g.	Density	Total nitrogen g./100 ml.	CaO g./100 ml.	P_2O_5 g./100 ml.	Cl g./100 ml.	Moisture g./100 g.	Total nitrogen g./100 g.	CaO g./100 g.	P_2O_5 g./100 g.	Weight of curd (lb.)
1	Milk	—	875·0	—	13·90	—	0·605	0·179	0·215	—	—	—	—	—	—
	Starter	—	13·1	—	12·76	—	—	—	—	—	—	—	—	—	—
2	Whey at running	120	706·3	0·165	6·91	1·020	0·183	0·077	0·105	—	51·25	2·71	0·745	0·804	—
3	Running to drying	120–140	37·0	0·23	7·88	1·015	0·198	0·112	0·123	—	43·96	3·12	0·861	0·975	—
4	After drying	140–155	8·5	0·28	7·57	1·019	0·207	0·107	0·141	—	40·26	3·32	0·903	0·967	—
5	After drying	155–175	4·0	0·34	7·16	1·022	0·215	0·172	0·161	—	—	—	—	—	—
6	After drying	175–265	2·38	0·46	7·61	1·021	0·240	0·258	0·200	—	39·17	3·39	0·916	1·02	109 (at milling)
7	Milling to salting	265–325	0·13	—	12·50	—	0·414	0·647	—	—	38·97	3·42	0·939	1·04	—
8	Salting to pressing	325–355	4·0	0·71	22·27	1·130	0·267	0·329	0·281	9·80	36·26	3·46	0·923	1·02	104·1 (to press)
9	Press whey	355–390	Aqueous* 3·5 Fat 1·0	0·85	16·78	1·067	0·185	0·356	0·291	4·55	—	—	—	—	—
10	Press whey	390–1725	Aqueous* 0·53 Fat 0·4	—	14·78	1·054	0·295	0·386	0·313	3·32	34·29	3·72	0·97	1·10	97·9 (cheese at 1 day old)

* Analyses on aqueous portion only.

A sample of skim milk was divided into three portions of which one was used raw, a second pasteurised for 30 min. at 142° F., and a third heated in a bacteriological steamer for 30 min. To a number of 10 ml. portions of each sample $N/10$ H_2SO_4 was added in the quantities 0, 0·5, 1·0, 1·5 ml., together with sufficient water to bring the volume added to 1·5 ml. in each case. Three pairs of tubes were made up with each concentration of acid. The first pair were titrated immediately, the second after 2 hours, and the third after 20 hours. In the intervening time samples were kept in the refrigerator. Controls showed no appreciable rise in acidity in this time. The results showed that with the addition of small quantities of sulphuric acid, the acid is recoverable in all cases within experimental error.

Table IV. *Weights of mineral constituents in whey and curd.*

Description	Total wt. lb.	Water lb.	CaO lb.	P_2O_5 lb.
Milk + starter	888	764·6	1·554	1·874
Whey 2	706·3	657·5	0·537	0·728
„ 3	37·0	34·1	0·041	0·045
„ 4	8·5	7·9	0·009	0·012
„ 5	4·0	3·7	0·007	0·006
„ 6	2·4	2·2	0·006	0·005
„ 7	0·1	0·1	0·001	—
„ 8	4·0	3·1	0·012	0·011
„ 9	4·5	3·8	0·012	0·010
„ 10	0·9	0·8	0·002	0·002
Weight lost in whey by milling	758·2	705·4	0·600	0·796
Weight in curd (calc.)	129·8	59·2	0·954	1·078
Weight in curd (found)	109·0	42·7	0·998	1·112
Difference	− 20·8	− 16·5	+0·044	+0·034
Weight lost in whey by pressing	762·3	708·6	0·613	0·807
Weight in curd (calc.)	128·1*	56·0	0·941	1·067
Weight in curd (found)	104·1	37·7	0·961	1·061
Difference	− 24·0	− 18·3	+0·020	−0·006
Weight lost in whey by 1 day	767·7	713·2	0·627	0·812
Weight in cheese (calc.)	122·7*	51·4	0·927	1·062
Weight in cheese (found)	97·9	33·6	0·950	1·077
Difference	− 24·8	− 17·8	+0·023	+0·015

* Allowing for 2·4 lb. salt added.

The experiment was repeated using lactic and acetic acids. The concentration of the acid used was 0·4 N and the quantities added were 0, 0·2, 1·0, 3·0 ml., together with water to bring the volume added to 3·0 ml. The results with the addition of 1 ml. of 0·4 N organic acids almost always gave a recovery of acid slightly greater than the amount added. This increase was of the order of 0·2 ml. of $N/10$ acid and occurred too regularly to be ascribed to experimental error. The recovery in the other cases was quantitative.

No regular variations were observed in either part of the experiment between raw, pasteurised and steamed milk.

A study was then made of the effect of increased acidity in the milk before renneting on the mineral content of the whey and of the relationship between the acidity of the milk and that of the whey derived from it.

A number of 300 ml. portions of skim milk, obtained from fresh morning's

milk, were measured into beakers and varying quantities of normal hydrochloric acid were added, together with sufficient water to bring the added volume up to 10 ml. in each case. The acid and water were mixed before addition and were added with constant stirring. After removal of 10 ml. of each milk for acidity determination, the samples were brought to 30° C. and 1 ml. of rennet solution (1 ml. rennet to 14 ml. water) was added. After 50 min. the curds were cut vertically by means of a cylinder of tinned iron (which fitted the beakers fairly closely) and across the mouth of which were stretched wires dividing it into 1 cm. squares. The curds were then stirred up with a palette knife and the whey was poured off immediately it separated through a funnel containing glass wool. Each 50 ml. portion of whey was collected and analysed separately. No appreciable increase in acidity was noted in the successive samples of whey from any one sample of curd, and the later portions had practically the same composition as the earlier portions. The results for

Table V. *Relationship of initial acidity of fresh milk to composition of rennet wheys.*

Analysis of milk: CaO 0·171 %; P_2O_5 0·244 %.

No.	Vol. milk ml.	Vol. *N* HCl added ml.	Vol. water added ml.	Acidity (ml. *N*/10 NaOH to 10 ml. sample)			Total solids	Density	Cl	CaO	P_2O_5
				Milk	Whey	Acidity of milk less acidity of whey					
1 and 2	300	—	10·0	1·88	1·13	0·75	6·57	1·025	0·102	0·062	0·123
3 and 4	300	0·5	9·5	2·03	1·23	0·80	6·56	1·026	0·110	0·067	0·125
5 and 6	300	1·0	9·0	2·14	1·37	0·77	6·56	1·024	0·114	0·070	0·127
7 and 8	300	1·5	8·5	2·30	1·51	0·79	6·54	1·025	0·120	0·073	0·129
9 and 10	300	2·0	8·0	2·42	1·63	0·79	6·49	1·026	0·125	0·076	0·133
11 and 12	300	4·0	6·0	3·04	2·09	0·95	6·65	1·025	0·150	0·090	0·143
13 and 14	300	6·0	4·0	3·76	2·61	1·15	6·63	1·025	0·169	0·105	0·156
15 and 16	300	8·0	2·0	4·30	3·15	1·15	6·78	1·026	0·191	0·127	0·171

the individual portions of each whey are therefore not reproduced here and only the mean values for each whey are given (Table V).

From Fig. 3 it will be seen that there is a linear relationship between the titratable acidities of both milk and whey and the amount of acid added to the milk. The line for milk is drawn at the calculated slope and shows a good agreement with the points plotted from individual determinations, indicating that the increase in acidity of the milk is directly proportional to the amount of acid added. The slope for whey, however, is less steep, showing that the acidity of the whey increases less rapidly than would be expected from the amount of acid added to the milk.

The change in calcium, phosphorus and chlorine content of the wheys with increased acidity in the milk is shown graphically in Fig. 4, in which all concentrations are expressed as milligram equivalents per 100 ml. The graphs for chloride found in the whey and chloride added as hydrochloric acid are parallel and thus indicate that the rise in chloride content of the wheys is

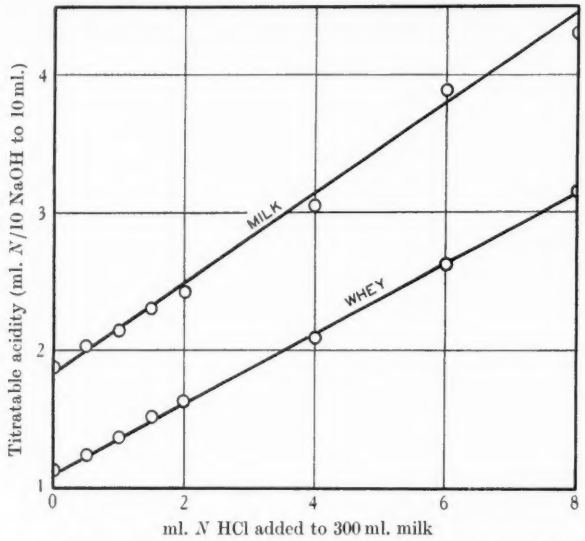

Fig. 3. Effect of addition of acid to milk on acidity of milk and whey.

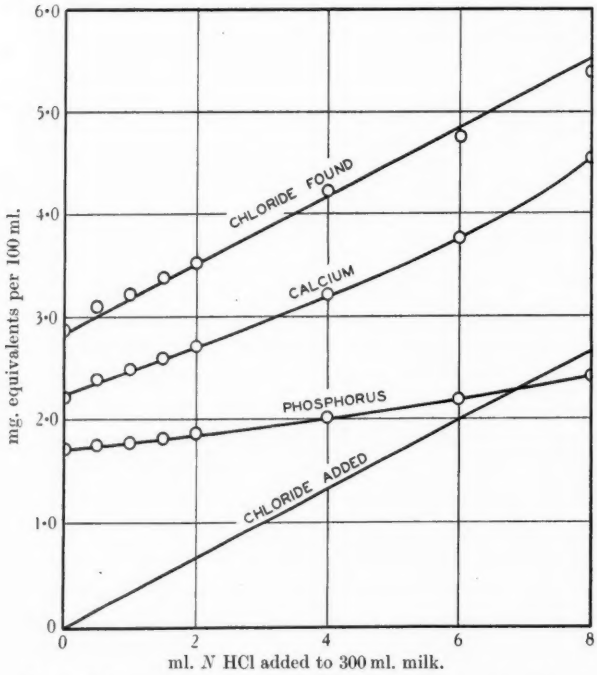

Fig. 4. Effect of addition of acid to milk on calcium and phosphorus content of whey.

quantitatively accounted for, within experimental error, by the amount of hydrochloric acid added to the milk. In the drawing of the graph for "chloride added", allowance has been made for the increase in chloride concentration in the whey due to removal of part of the solids of the milk as curd.

The phosphorus values reported include, of course, organic phosphorus, but it seems reasonable to suppose that any increase in phosphorus concentration is due to inorganic phosphate. Fig. 4 shows a slow rise in phosphate concentration with increase in acidity, while the rise in calcium concentration is much more rapid, only slightly less than one equivalent of calcium going into the whey for each equivalent of acid added. If the acid acted only on the calcium caseinate and calcium phosphate of the milk, each equivalent of acid added would cause one equivalent of calcium to go into solution as chloride, while a further quantity of calcium would appear as soluble monocalcium phosphate. The explanation given by Grimmer and Paape(6) in discussing their results on milk souring appears to apply also to the results of the present investigation: there are substances in milk, other than casein and calcium phosphate, which combine with acid. The citrates and alkali phosphates present in milk would be sufficient to neutralise quite an appreciable quantity of acid.

A repetition of the experiment confirmed the results obtained.

Effect of acidity on the mineral content of cheese.

The pronounced effect of the acidity of the milk before renneting in controlling the escape of calcium and phosphorus in the whey was further investigated on the factory scale, in conjunction with a series of experiments carried out under the direction of Prof. Riddet at the Institute Factory, dealing with the effect on the general cheesemaking procedure of adding dilute hydrochloric acid to the milk at setting. In each case a sufficient quantity of normal acid was used to raise the acidity of the milk to 0·25 per cent. (calculated as lactic acid). Three vats were run on each day. The acid was added to vat 3, an equal volume of water was added to vat 2, while vat 1 was a control with undiluted milk. The whole of the milk supply was mixed in a large vat and then flash pasteurised at 155° F. before being divided between the three experimental vats. The same proportions of starter, rennet and salt were used in each of the vats. The experiment was repeated 25 times. Table VI shows the results of analysis of the whey samples from one day's make in this experiment. The increased losses of calcium and phosphorus in the early wheys from the vat with added acid bear out the evidence of the earlier experiments.

Determinations of moisture, fat and calcium were made on all the cheeses 9–12 weeks after manufacture. The whey acidities and analyses of the cheese for a typical day's make are given in Table VII.

There was seldom any appreciable difference between the behaviour of vats 1 and 2. The acidity in vat 3 was invariably higher in the whey at running than in vats 1 and 2, but the difference decreased after the drying stage and,

as illustrated in Table VII, the acidity of the wheys from all vats was usually practically the same at salting. On a few occasions the maker reported that the curd of vat 3 was rather too soft and tender between milling and salting and appeared to be holding a fair amount of moisture, while the curds of vats 1 and 2 were considerably firmer.

The average moisture content and content of calcium in the fat-free dry matter in the cheese from each vat over the 25 days are given in Table VIII.

The calcium in the vat 3 cheese was always lower than that in the cheeses from vats 1 and 2.

Table VI. *Effect of initial acidity of milk on mineral composition of whey.*

	Acidity/vol.		P_2O_5/vol.		CaO/vol.	
Sample	Vat 2	Vat 3	Vat 2	Vat 3	Vat 2	Vat 3
Milk (acidity taken at renneting)	0·20	0·27	0·269	0·268	0·174	0·171
Whey, 10 min. after cutting	0·125	0·185	0·143	0·158	0·059	0·067
Whey at running	0·19	0·23	0·150	0·166	0·066	0·092
Whey at drying	0·27	0·28	0·169	0·183	0·106	0·112
Whey, 30 min. after drying	—	—	0·204	0·211	0·167	0·183
Whey at milling	—	0·95	—	—	—	—
Whey at hooping	0·92	0·85	—	—	—	—

Table VII. *Details of manufacture of control cheese and of experimental cheese from milk with acid added.*

			Acidity/vol. at							Analysis at 10 weeks		
Vat No.	Wt. of milk (lb.)	Quantity of water or acid added	Ren-neting	Cutting	Running	Drying	Milling	Salting	Hooping	Mois-ture	Ca	Ca in fat-free dry matter
1	872	Control	0·195	0·13	0·195	0·27	0·76	1·00	0·85	35·32	0·64	2·18
2	800	2900 m.l. water	0·195	0·13	0·195	0·28	0·82	1·00	0·85	34·27	0·65	2·19
3	800	2900 m.l. *N* HCl	0·27	0·185	0·225	0·29	0·80	1·00	0·80	34·42	0·61	2·08

Table VIII.

Cheese	Moisture %	Ca in fat-free dry matter %
Vat 1	34·13	2·30
„ 2	33·76	2·32
„ 3	34·75	2·10

The raising of the acidity of the milk prior to renneting, though it does not noticeably affect the acidities in the later stages of the process, definitely causes a sufficient loss of minerals in the early wheys to give a markedly lower calcium content in the cheese. This is in agreement with the results of Wode (3) and the conclusions of Nottbohm and Baumann (7) and McCann, Caulfield and Kramer (8), though in the two latter investigations the conclusions were based on comparisons of cheeses made under widely different conditions. The effect of the higher acidity at setting on the extent of calcium loss may be, in part at any rate, the explanation of the necessity for an adequate ripening period before the addition of rennet. The effect of excessive acidity during the making

process in causing a rapid breakdown of the cheese, and frequently of imparting a bitterness to the cheese, may also be related to the resultant changes in mineral content.

Discussion.

The rise found in concentrations of calcium and phosphorus in the wheys throughout the cheese-making process is in general agreement with the results of Lloyd. No previous investigators, however, appear to have observed the sudden temporary fall noted after salting in the present investigation. This fall in concentration of mineral constituents should afford an explanation of the drop in acidity frequently noticed in practical cheese making, since the buffer action of the calcium phosphate in the whey will contribute largely to the titratable acidity. The relationship between the mineral content of wheys and their buffer value will be dealt with in a second paper.

A comparison may be made of the results for the percentages of the minerals of milk retained in the cheese when made under normal conditions. Lloyd (1) states that on the average approximately one-third of the mineral matter of milk is retained in the cheese. McCann, Caulfield and Kramer (8) from analyses on a number of American Cheddar cheese find that the average percentages retained are 80·4 per cent. for calcium and 38·1 per cent. for phosphorus. From the results given in Tables III and IV of the present paper the figures are found to be 60 and 57 per cent. respectively.

A complete picture of the mineral changes in Cheddar cheese making could only be obtained if, simultaneously with the determinations cited above, analyses were made of the potassium, sodium, magnesium, citric acid and lactic acid contents of the wheys. It has not been possible to deal with the preliminary operations of such an extensive series of analyses in one day, as would be necessary to avoid further bacteriological action in the samples, but some isolated investigations have indicated that there is a distinct rise in the sodium and potassium content of cheese whey after drying and in the citric acid content throughout the process. The latter finding is contrary to the results of other workers. Bosworth and Prucha (13), for example, found that citric acid had entirely disappeared from both curd and whey by the time the cheese was hooped. In experiments with milk to which a starter culture was added, citric acid had entirely disappeared in 60 hours, though there was no appreciable decrease in its concentration till 28 hours, by which time the milk was curdled. Bosworth and Prucha ascribed the fermentation to *B. lactis aerogenes*. Hammer (14) has also shown that citric acid is attacked by organisms (*Str. citrovorus* and *Str. paracitrovorus*) found in commercial starter cultures. According to Lampitt and Bogod (15) citric acid is completely fermented by *Str. lactis* and *Bact. coli* in one or two days.

Apart from the results of Bosworth and Prucha on whey, however, the general evidence appears to indicate that the fermentation of citric acid by starter bacteria is slow and does not begin till a considerable amount of acid is developed.

The results for sodium and potassium and for citric acid require further confirmation and are therefore not reproduced here in detail. The changes in lactic acid and lactose concentration in curd, whey and curd juice will be discussed in a third paper.

SUMMARY.

1. Analyses have been made of the wheys obtained at various stages in the manufacture of Cheddar cheese.

2. There is shown to be a steady rise in the concentrations of calcium and phosphorus in the wheys throughout the process up to salting. The calcium rises much more rapidly than the phosphorus.

3. After salting there is a sudden temporary fall in the concentration of both calcium and phosphorus in the whey, the calcium fall being particularly pronounced.

4. The concentration of calcium and phosphorus in the whey vary in accordance with the titratable acidity of the whey.

5. In normal cheese making some 60 per cent. of the calcium and 57 per cent. of the phosphorus in the milk is retained in the cheese.

6. Of the minerals lost in the whey some 86 per cent. of the calcium and 90 per cent. of the phosphorus escape before running.

7. An increase in the acidity of the milk before renneting increases the loss of minerals in the whey and reduces the calcium content of the cheese.

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108. STUDIES ON THE CHEMISTRY OF CHEDDAR CHEESE MAKING

II. THE BUFFER CAPACITY OF WHEYS

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(With 5 Figures.)

In a previous paper (1) some anomalies in results of acidity determinations during the cheese-making process were discussed and an attempt was made to relate these to variations in the mineral content of the wheys. In the present section of the investigation, determinations have been made of the buffer capacity of wheys and the results have been related to their mineral content. A definite connection between the mineral content of whey and its titratable acidity is thus established.

A number of investigators have studied the buffer system of milk, and several (Whittier (2), Wurster (3) and Oldenburg (4)) have also worked on wheys. These wheys, however, were prepared specially for comparison with the milk. No investigations appear to have been made on the buffer capacity of wheys obtained during the cheese-making process.

Experimental.

*p*H determinations were made with a Cole direct-reading potentiometer using the quinhydrone electrode in conjunction with a quinhydrone reference cell. Gold electrodes were used in both half-cells. Measurements were carried out at room temperature (14–16° C.) and the temperature was noted at frequent intervals and corrections applied. The acid and alkali used in the titrations were 0·5 *N* and were added to the whey-quinhydrone system from microburettes. After each addition the liquid was stirred and the *p*H determined. Separate portions of the sample (20 ml. in each case) were used for the titrations with acid and with alkali. The time occupied in each half of the titration was 10–15 min. The potential drift of the electrode during this time was found to be negligible.

It has been shown by Whittier (2) that, in the determination of the titration curve of milk, a time of 1½–2 hours is required for the attainment of equilibrium between the added acid or alkali and the salts of milk. Even greater difficulty was experienced by Holt, La Mer and Chown (5) in work on calcium phosphate solutions, with some of which 6 months' intermittent shaking failed to produce complete equilibrium, though 10 days' shaking produced a fair approximation to equilibrium.

The method adopted by Whittier was to make each *p*H determination on a separate sample. The appropriate quantity of acid or alkali was added from a pipette and 2 hours allowed for the attainment of equilibrium. To prevent bacterial action during this period 1 drop of formalin was added to 100 ml. of milk. This reagent was shown to have no appreciable effect on the *p*H, but it was not demonstrated that it had no influence on the buffer capacity of the protein.

Whittier's method also has the disadvantage of requiring a considerable volume of sample for the number of *p*H determinations involved. In the later stages of the cheese-making process prior to salting, little whey is obtainable from vats of the size in use at this Institute. Buffer estimations in which a considerable time is allowed for the attainment of equilibrium would not be strictly comparable with acidity determinations in which the time occupied is very short.

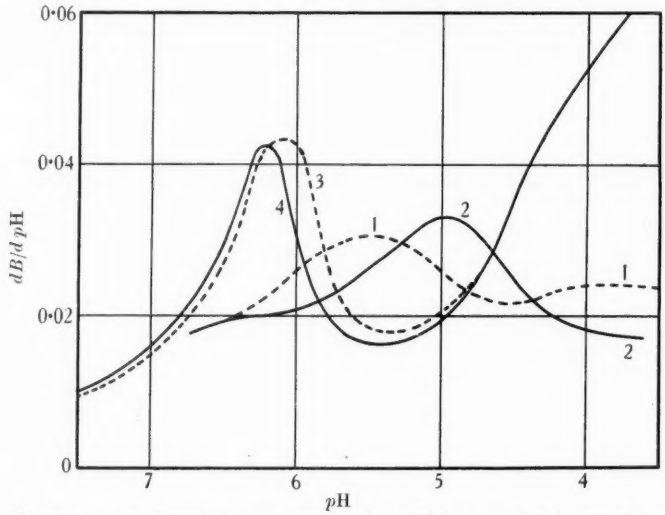

Fig. 1. Effect of time allowed for attainment of equilibrium on buffer capacity of whey. 1, milk, *p*H determinations after 2 hours; 2, milk, *p*H determinations immediately; 3, whey, 35–50 min. after drying, *p*H determinations after 2 hours; 4, whey, 35–50 min. after drying, *p*H determinations immediately.

A comparison of the method of Whittier and that in use in the present investigation was made on a sample of milk and a sample of whey obtained 35–50 min. after drying—the latest stage at which a sufficient volume of whey was available. Buffer curves for the two samples by both methods are given in Fig. 1. These show that, if insufficient time is allowed for attainment of equilibrium, a considerable error in buffer value occurs with milk in the *p*H zone where casein coagulates. With whey there is also an error, but it is smaller and does not greatly affect the form of the curve or the height or position of the

buffer maximum. It was considered that results of sufficient accuracy for the present purpose could be obtained on wheys by successive additions of the titrating solution to the same portion of the sample without allowing a lengthy period for reaching equilibrium.

In the calculation of buffer values ($dB/d\ p$H) the method of Whittier was used:

$$\frac{dB}{d\ p\text{H}} = \frac{\text{(m.l. acid or base added to produce } p\text{H change) (normality factor of acid or base)}}{\text{(average vol. of sample over range involved) (}p\text{H change produced)}}.$$

The resulting value is attributed to the pH value at the mid-point of the range involved.

As noted by Whittier and by Oldenburg, who followed Whittier's methods, where $dB/d\ p$H is calculated for small increments of acid or alkali any experimental error is magnified to such an extent that a very irregular curve results. In the present investigation, values of pH and of acid or alkali added were read off a smoothed titration curve plotted from experimental results. This method seemed preferable to that of the previous investigators, who took only a few experimental points at wide intervals.

The samples of whey for buffer estimation were obtained from the factory of the Institute during the course of the manufacture of normal Cheddar cheese. Buffer determinations were made on whey on several days at intervals during the season, and a general similarity was found between the various sets of results. Accordingly the results of only 1 day's experiments are reproduced here. They are shown in Fig. 2 as titration curves and again in Fig. 3 as curves of buffer value against pH. The curves for the milk used, though somewhat less accurate owing to incompleteness of equilibrium in the titration, is included for comparison.

These results show that whey obtained shortly after cutting has but little buffering power. Later wheys have increasingly greater buffering power, both at low pH values, where the curves rise steadily, and in the vicinity of pH 6, where a maximum buffering occurs. This maximum, which first appears in whey at running at pH 6·5, rapidly increases in intensity and also shifts slightly towards the acid side reaching pH 5·8 between milling and salting in the wheys investigated. After salting there is a reduction in buffering power over the whole range and a considerable change in the form of the curve.

In an endeavour to assign definite roles to the various buffer substances present in whey, buffer curves were determined for a number of the constituents of whey. Whey protein was not included in this investigation, in view of the work of Wurster (3), who has shown that the heating of milk or whey has no appreciable influence on the buffer value and hence concluded that the heat-coagulable proteins have but little buffering power.

The buffer curves for the various substances investigated are shown in Fig. 4. The curve for lactic acid at pH values below 5 practically coincides with that for whey at milling over the same range. The lactic acid content of this whey (about 1·5 per cent.) would be somewhat less than that of the lactic

acid solution used (2 per cent.), but we may conclude that lactate is responsible for the greater part of the buffering power of whey over this range. Whittier came to a similar conclusion for lactic acid whey containing 0·5 per cent. of lactic acid.

The curve for calcium phosphate shows a maximum at a *p*H corresponding closely with that of the buffer maximum in wheys. The *p*H of this maximum (about *p*H 6) is lower than that of the buffer maximum for phosphoric acid or soluble phosphates (*p*H 6·85). This is due to the complicating factor of the

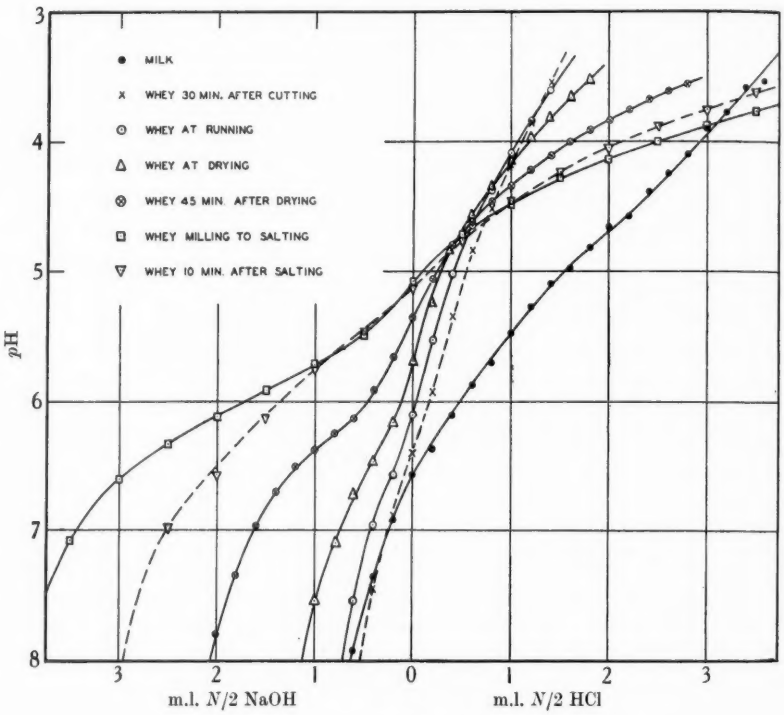

Fig. 2. Titration curves of milk and wheys

formation of insoluble di- or tricalcium phosphate from the soluble monocalcium phosphate as the solution becomes more alkaline. The theory of this aspect of calcium phosphate buffering has been developed by Kugelmass (6) and by Holt, La Mer and Chown (5). Addition of alkali to an acid solution of calcium phosphate will give a titration curve identical with that for phosphoric acid until a point is reached at which the first equivalent of phosphoric acid is neutralised. Further addition of alkali will produce dicalcium phosphate which is much less soluble than the monocalcium phosphate. Precipitation will occur, either of the dicalcium phosphate or of tricalcium phosphate formed

from this, leaving only monocalcium phosphate in solution. As a result, addition of alkali will not increase the *p*H but will merely precipitate calcium. When the whole of either the calcium or the phosphate in solution has been precipitated the *p*H will rise rapidly. The insoluble nature of the di- and tricalcium phosphates thus gives rise to a strong buffer effect of a different nature from the buffering found in solutions of soluble phosphates.

The *p*H at which maximum buffering occurs will depend on the concentration of calcium and phosphate in the solution and on the solubility product

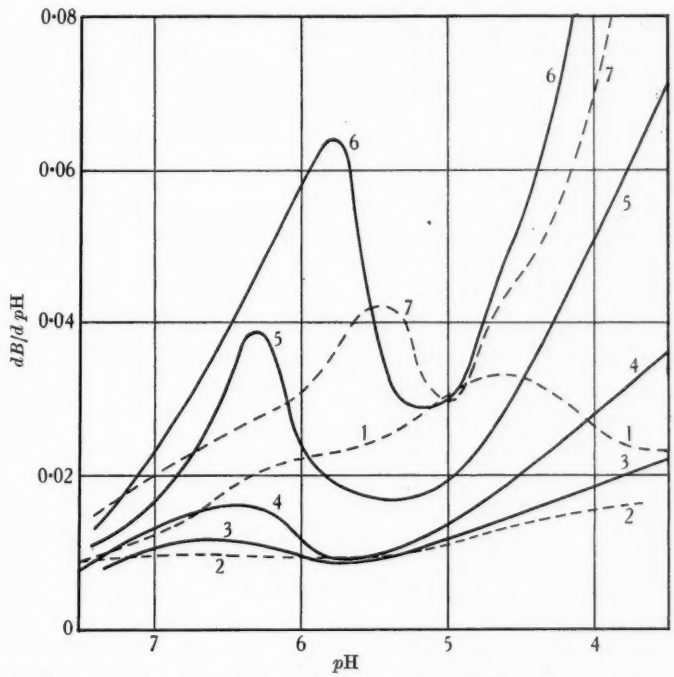

Fig. 3. Buffer curves for milk and wheys. 1, Milk; 2, whey, 30 min. after cutting; 3, whey at running; 4, whey at drying; 5, whey, 45 min. after drying; 6, whey, milling to salting; 7, whey, 10 min. after salting.

of the calcium phosphate precipitated. This solubility product decreases slightly with rise in temperature but is greatly increased by the presence of certain salts. Holt, La Mer and Chown have shown that sodium chloride and magnesium sulphate increase the solubility product and hence decrease the buffering. Citrates also exercise a marked effect on the solubility product. The influence of citric acid on the buffering is illustrated in curve 4 of Figs. 4 and 5. The addition of 0·3 per cent. of citric acid to a solution of monocalcium phosphate containing 0·3 per cent. calcium changes the nature of the buffer curve considerably. The buffer maximum is lower and now occurs at *p*H 6·8, closely

approaching the value for soluble phosphates (6·85). This concentration of citric acid evidently increases the solubility product of tricalcium phosphate to such an extent that the calcium phosphate remains almost completely in solution at the concentration present.

The concentration of citric acid in whey (of the order of 0·15 per cent.) is less than that used in this experiment and is evidently insufficient to suppress

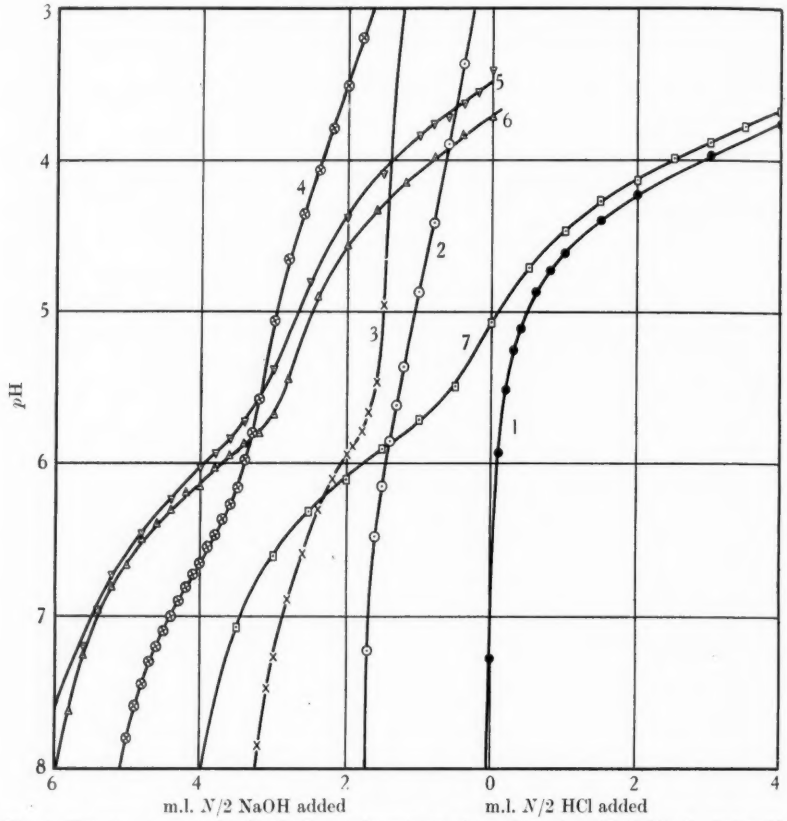

Fig. 4. Titration curves of individual constituents of wheys. 1, lactic acid (2 %); 2, citric acid (0·3 %); 3, mono-calcium phosphate + HCl (0·3 % Ca); 4, calcium phosphate (0·3 % Ca) acid (0·3 %); 5, "synthetic whey"; 6, "synthetic + citric whey" + 16 % sodium chloride; 7, whey, milling to salting.

the calcium phosphate buffering to any great extent. In milk, however, the citric acid present, together with the low concentration of soluble calcium and phosphate, is able completely to prevent the formation of a buffering maximum in the region of *p*H 6.

The buffering of 0·3 per cent. citric acid alone is shown in curve 2 of Figs. 4 and 5. Evidently in the concentrations in which citric acid is present in whey

it contributes but little to the buffering, though it probably does influence the calcium phosphate buffer maximum to some extent.

These experiments indicate that in wheys obtained before salting, the buffering at low *p*H values is chiefly due to lactate, while that at higher *p*H values is mainly due to calcium phosphate. In order to confirm this theory more completely a "synthetic whey" was prepared. This was made by shaking

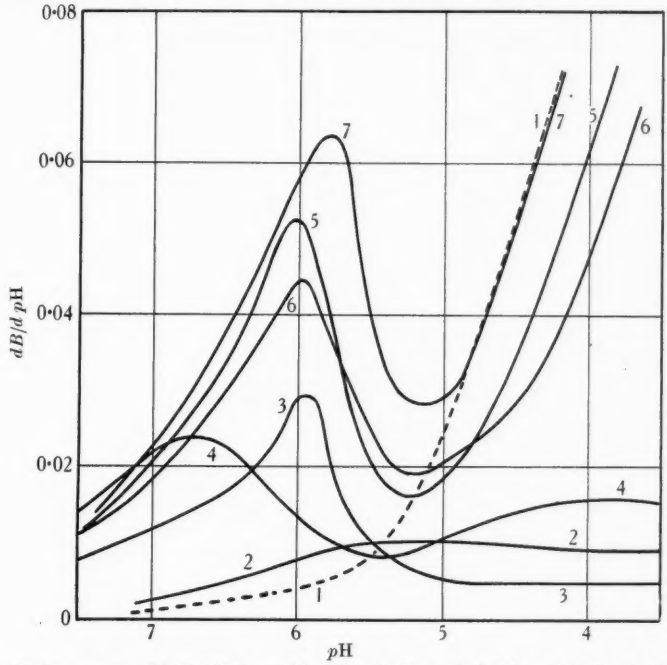

Fig. 5. Buffer curves of individual constituents of wheys. 1, lactic acid (2 %); 2, citric acid (0·3 %); 3, monocalcium phosphate + HCl (0·3 % Ca); 4, calcium phosphate (0·3 % Ca) + citric acid (0·3 %); 5, "synthetic whey"; 6, "synthetic whey" + 16 % sodium chloride; 7, whey, milling to salting.

monocalcium phosphate, calcium lactate, citric acid and lactic acid with water for some hours. It was made up to contain:

Ca	... 0·35 per cent.	Citric acid	... 0·15 per cent.
P_2O_5	... 0·35 per cent.	Lactic acid	... 1·5 per cent.

These concentrations correspond approximately with the average composition of wheys at milling. The curve for this "synthetic whey" is given in curve 5 of Figs. 4 and 5. The agreement between this and curve 7—a whey obtained between milling and salting—is very satisfactory. The general form of the curves is similar. The slightly lower buffering of the "synthetic whey" is no doubt due to slight differences in concentration and to the omission of other constituents of whey which will also contribute to a small extent to the buffering.

The analyses of wheys reported in the first paper of this series (1) show that calcium and phosphorus as well as lactic acid increase steadily in concentration from cutting to salting. This increase runs parallel with the increase in buffering due to lactate and phosphate as shown in the buffer curves. With increase in the concentration of calcium phosphate the buffer maximum will be higher and will occur at a slightly lower *p*H, since a smaller proportion of the calcium will have to be converted to di- or tricalcium phosphate to produce saturation.

The form of the curve for whey obtained after salting (Fig. 3, curve 7) has not been completely explained. The mere addition of sodium chloride has but little effect on the buffering as shown in Fig. 4, curve 6—synthetic whey containing 16 per cent. sodium chloride. After salting there is a drop in the concentrations of calcium and phosphorus in whey, but these concentrations do not fall in the same ratio. This change in the proportions of the constituents, together with the possible entry of fresh buffer substances such as brine-soluble proteins, may afford an explanation of the buffer curve.

The results of this investigation have shown that in determining titratable acidity of whey it is the calcium phosphate buffer system which is titrated while the lactate present will have little buffer effect over the *p*H range involved.

The amount of calcium phosphate present and hence the amount of buffering will, of course, depend on the development of lactic acid, so that titratable acidity is connected, though indirectly, with percentage of lactic acid. The indirect nature of this connection will, however, permit the entry of disturbing factors which may give rise to anomalous acidity results.

SUMMARY.

1. The buffer values of wheys obtained at various stages of the cheese-making process have been determined over the *p*H range 3·5–7·5.

2. The buffering between *p*H 3 and 5 is shown to be chiefly due to lactate, while that at higher *p*H values is chiefly due to calcium phosphate.

3. The results of the buffer determinations are shown to be in accord with the analyses of the mineral constituents of wheys reported in the first paper of this series (1).

ACKNOWLEDGMENTS.

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109. STUDIES ON THE CHEMISTRY OF CHEDDAR CHEESE MAKING

III. THE CONVERSION OF LACTOSE INTO LACTIC ACID BY STARTER CULTURES

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(With 4 Figures.)

The rate of transformation of lactose into lactic acid is of very great importance in the cheese-making process, since it controls the changes in mineral composition and in the texture and other physical properties of the curd. As an indication of the rate of transformation, determinations of the titratable acidity of the whey expelled from the curd have for many years been used by the cheese maker. In spite of its importance, however, the lactose-lactic acid relationship has received only scant attention from a strictly quantitative point of view, probably to a large extent on account of the difficulties involved in the accurate estimation of lactic acid. The development within recent years of methods which permit a reasonably accurate recovery of lactate from biological fluids has removed the latter difficulty.

In the succeeding paper (Part IV) of this series, an account will be given of an attempt to define in a quantitative manner the lactose-lactic acid relationship throughout the cheese-making process. As a preliminary to this investigation, it was considered desirable to study this reaction in the souring of milk to which a starter culture had been added. While the conditions of growth in milk are admittedly different from those obtaining in cheese curd, it was thought that such an investigation would give some indication of the extent to which substances other than lactic acid are likely to be formed in the fermentation of lactose in the cheese vat.

Van Slyke and Bosworth (1), in experiments on the souring of milk to which a culture of *B. lactis acidi* and *B. lactis aerogenes* had been added, found that only 70–93 per cent. of the lactose fermented was converted to lactic acid. According to these authors, products other than lactic acid are formed from lactose, varying in kind and amount according to the conditions of the fermentation process. The lactic acid was estimated by isolation of the zinc salt, which is admittedly a very uncertain method of recovery.

Orla-Jensen (2) stated that the true lactic acid bacteria form only traces of succinic acid and volatile acids (acetic, propionic and carbon dioxide), and that the total acid formed could be calculated as lactic acid without essential

error. In a state of abnormally high vitality the bacteria could, however, produce appreciable quantities of carbon dioxide, while under unfavourable conditions they could produce an increased quantity of acetic acid. The hydrolysis of lactose to glucose and galactose was caused by only a few bacteria and then mostly in old cultures containing many dead cells.

Lampitt and Bogod (3) have presented an extensive review of the literature, and results of experiments with a number of species of bacteria, showing the relation between their period of incubation in milk, and the resultant titratable acidity and lactic acid contents of the milk. Appreciable quantities of lactic

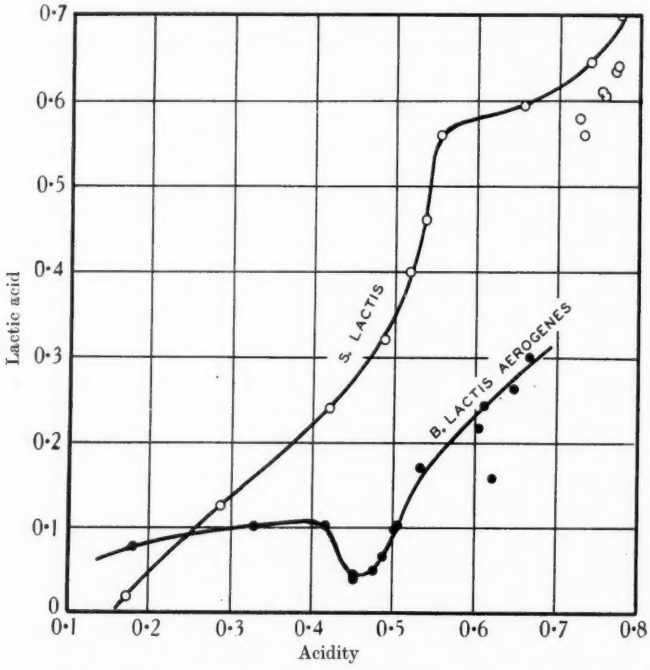

Fig. 1. Relation between titratable acidity and lactic acid (Lampitt and Bogod).

acid were formed by only two of the species investigated, viz. *Streptococcus lactis* and *B. lactis aerogenes*. Their results, however, have been studied only from the standpoint of rate of change, *i.e.* each set of figures has been plotted against time of incubation, and in that form the results cannot be related to the subject of this paper. The curves in Fig. 1 have been drawn from the figures of Lampitt and Bogod. The curve for *Streptococcus lactis* indicates that in their experiments there was considerable production of lactic acid without much corresponding change in titratable acidity. The curve for *B. lactis aerogenes* indicated that the greater part of the acidity produced is due to acids other than lactic acid.

Experimental.

Methods of analysis.

The *titratable acidity* is recorded as a percentage by weight of lactic acid in the sample.

Lactose.

Lane and Eynon's volumetric procedure (4, 5) as modified by Farmer (6) was used. Since the modifications introduced by Farmer are not yet available in the general literature, the details are appended here.

(*a*) *Preparation of the sample.* A portion (20 ml.) was measured into a 200 ml. graduated flask containing 80 ml. of distilled water. Lead acetate (10 ml. of 10 per cent. solution) and saturated sodium fluoride (36 ml.) were added, the flask being shaken after each addition. Water was added to make the volume up to 200 ml., the mixture was allowed to stand for 1 hour and was then poured on to a dry filter, the filtrate being used in the titration.

(*b*) *Solutions used.* The copper sulphate solution (solution A) used (35·46 g. $CuSO_4.5H_2O$ per litre) was one-half the concentration of that specified by Lane and Eynon, while solution B (346 g. Rochelle Salt and 100 g. NaOH per litre) was of the same strength as that used by these authors.

(*c*) *Procedure.* 25 ml. of solution A were measured accurately with a pipette into a 250 ml. conical flask, and 12·5 ml. of solution B, measured in a cylinder, were added. A quantity of milk serum slightly smaller than that required to reduce the copper, was run in from a burette, and water was added to bring the final volume to approximately 75 ml. The flask was immersed to the level of the liquid in a boiling water bath. After 3 min. 20 sec. the flask was transferred to a wire gauze over a Bunsen flame adjusted so as to bring the liquid to the boil in 40 sec. When the boiling had continued for $1\frac{1}{2}$ min., *i.e.* $5\frac{1}{2}$ min. from the commencement of the analysis, 6 drops of 1 per cent. aqueous methylene blue were added, and at 6 min. milk serum was added from the burette at such a rate that the blue colour was discharged approximately 7 min. from the start of the experiment. (Observation of the end point was facilitated by the use of a ring of white asbestos paper on the wire gauze surrounding the flask.) The quantity of milk serum to be added at the outset was gauged from a preliminary rough titration, a margin of 0·5–1·0 ml. being allowed to ensure that the endpoint was not overrun.

For samples containing such a small percentage of lactose that more than 50 ml. of serum would be required in the titration, 30 or 40 ml. of the serum was added to the flask and the remainder of the lactose needed to complete the reduction was added as a 0·5 per cent. solution of lactose. In this way the final volume could be kept approximately equal to the set value of 75 ml.

Each batch of copper sulphate solution was standardised on a 0·5 per cent. solution of lactose and the concentration of lactose in the milk samples calculated by proportion from the titration figures. Tests on solutions of

lactose of various concentrations and on milk with various quantities of added lactose showed that the error of the determination did not exceed ± 1 per cent., while the agreement of duplicates was usually better than 0·5 per cent. Concentrations of lactose are expressed as percentage of lactose monohydrate ($C_{12}H_{22}O_{11}.H_2O$).

Lactic acid.

Protein and lactose were removed by successive treatments with copper sulphate and calcium hydroxide, and lactic acid was estimated on the final filtrate by oxidation to acetaldehyde with permanganate. The procedure described by Meyer(7) was followed up to the titration stage. The use of standard sodium bisulphite for the absorption of acetaldehyde and back titration of the bisulphite with iodine as used by Meyer did not give satisfactory results and the method of Clausen(8, 9) was employed. The acetaldehyde was absorbed by bisulphite, the excess of bisulphite was exactly removed with iodine and the acetaldehyde-bisulphite complex was then decomposed with sodium bicarbonate and the liberated bisulphite titrated directly with standard iodine. The recovery of lactate from solutions of zinc lactate was about 93 per cent., while on milk with added lactate the recovery was about 90 per cent. The results for milk and wheys were corrected (multiplied by 1·11) to allow for the incomplete recovery. The error in the estimation was then in the region of ± 5 per cent.

Conversion of lactose to lactic acid in milk.

Samples of skim milk were pasteurised at 65° C. for 20 min., cooled and 4–6 per cent. of a commercial starter culture added. The milk was incubated at 37° C., samples being removed periodically until the milk had clotted. The results of determinations of acidity, lactose and lactic acid in a typical experiment are shown in Fig. 2. The increase in acidity (expressed as lactic acid) is seen to be slightly greater than the amount of lactic acid formed, but is approximately proportional to it. The decrease in lactose, however, does not bear a linear relationship to the increase in lactic acid or acidity, and it appears that 20–25 per cent. of the lactose fermented is converted to substances other than lactic acid. In these experiments, however, owing to the rise in acidity, the formation of acid practically ceased when only about 10 per cent. of the lactose had been fermented and the later stages of the action were at a *p*H too low for the favourable working of the organisms.

Further experiments were carried out in which the acid formed was neutralised by periodical additions of alkali. Acidity development was allowed to proceed till a titratable acidity corresponding to about 0·5 per cent. of lactic acid had been developed. Sufficient 2*N* sodium hydroxide was then added to reduce the acidity to about 0·3 per cent. lactic acid. The experiment was continued in this way with periodical additions of alkali till acidity development had practically ceased and but little lactose remained. The periodical

additions of alkali produced a progressive dilution of the milk, and the position was further complicated by the removal of samples for the determination of acidity, lactose and lactic acid. A known volume of milk was taken for the experiments and the exact volumes of standard alkali added and of samples removed were noted. It was then possible to calculate the amount of the original milk contained in any sample and the total quantity of alkali which was present in that sample. All results from the various determinations were calculated as percentages on the original milk.

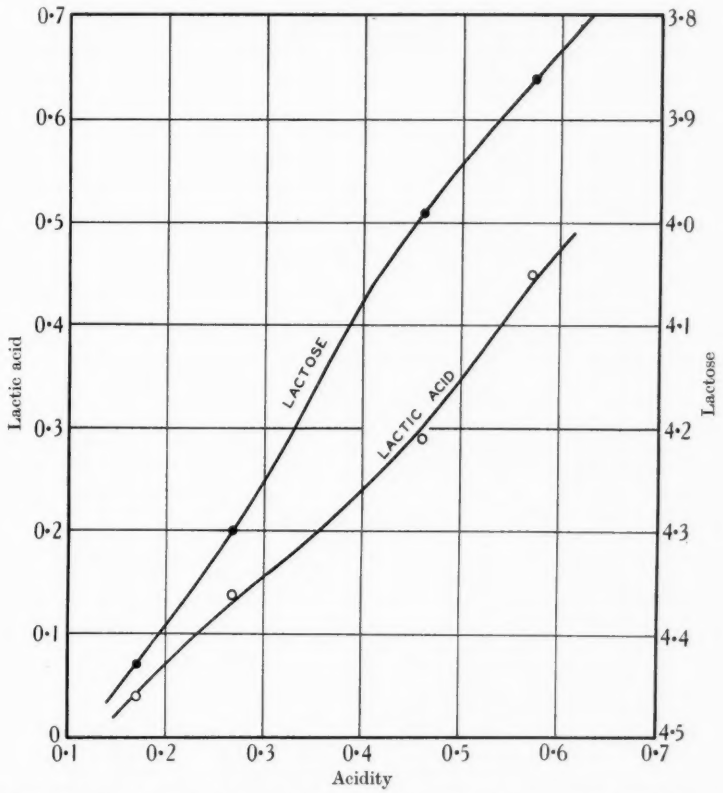

Fig. 2. Relation between lactose, lactic acid and acidity with commercial starter culture.

The results of one experiment are shown in Fig. 3. In this experiment 3 litres of milk pasteurised at 65° C. and cooled to 20° C. were treated with 110 ml. of a mixed starter culture and kept at 20 ± 1° C. The fermentation of the lactose required 7 days. During this period 594 ml. of alkali (1·97 *N*) were added and the final sample contained 77·2 per cent. of the original milk.

It is evident from the curves that during the early stages of the experiment the amount of lactose fermented was greater than the amount of lactic acid

formed. Taking the range of the experiment during which the first 1 per cent. of acid was formed, we have, reading from the curves: lactose decomposed, 1·22 per cent.; lactic acid formed, 0·99 per cent.; acidity increase, 1·00 per cent. Apparently over this section of the experiment some 19 per cent. of the lactose fermented was converted into substances other than lactic acid. Over the same range the increase in acidity was almost exactly equal to the percentage of lactic acid formed.

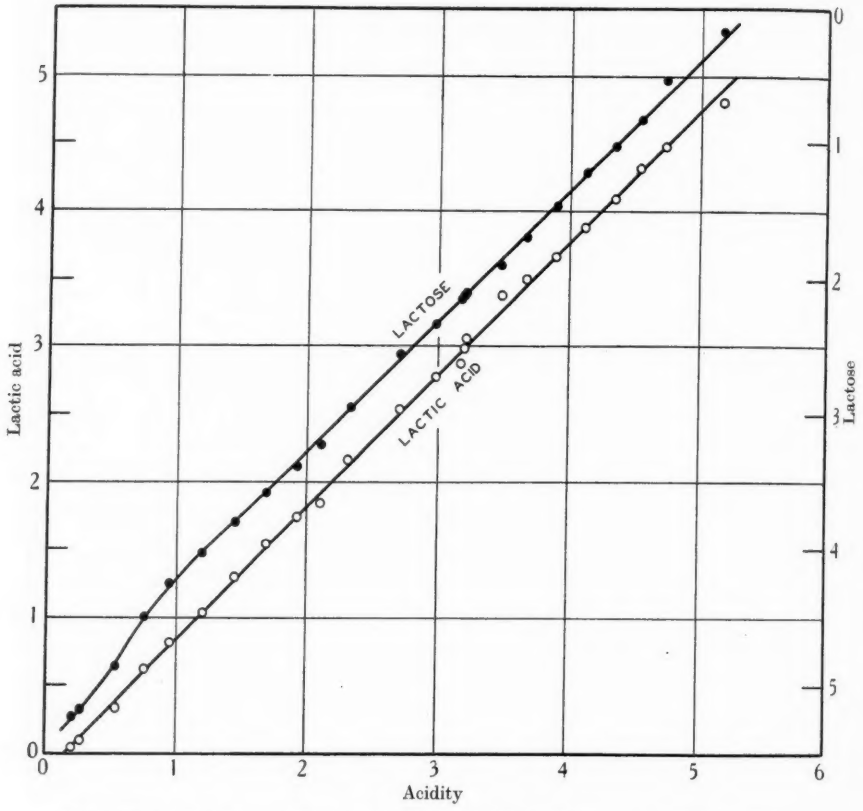

Fig. 3. Relation between lactose, lactic acid and acidity with commercial starter culture.

Over the remainder of the experiment the agreement between lactose decomposed and lactic acid formed was much closer. The figures from the curves are: lactose decomposed 3·87 per cent.; lactic acid formed 3·91 per cent.; acidity increase 4·02 per cent. The slight excess of lactic acid and acidity over the amount of lactose lost may not be significant as it is within the margin of experimental error.

Over the whole experiment we have: lactose decomposed, 5·09 per cent.; lactic acid formed, 4·90 per cent.; acidity increase, 5·02 per cent.

Another experiment was conducted on the same lines, but in this case the starter used was a pure culture derived from a single colony, and the whole of the sampling and other operations in the experiment were conducted in a sterile manner. The results of this experiment, which occupied 9 days, are shown in Fig. 4. There is a noticeable difference here in that the relation between lactose decomposed and acidity formed is linear throughout the whole course of the experiment, and the effect previously noticed in the early stages

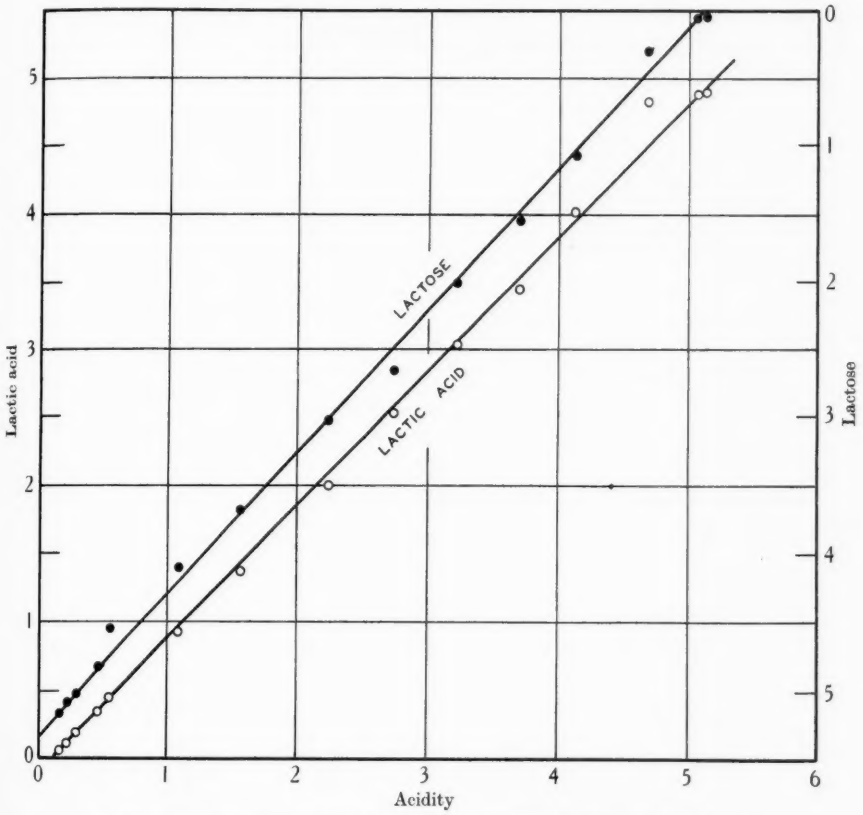

Fig. 4. Relation between lactose, lactic acid and acidity with pure culture starter.

of the fermentation is absent. The figures as read from the graph over the whole course of the experiment are: lactose decomposed, 5·20 per cent.; lactic acid formed, 4·97 per cent.; acidity increase, 4·99 per cent. There is a remarkably close correspondence between the increase in acidity and the amount of lactic acid formed throughout the experiment. It appears, however, that in this case, some 4 per cent. of the lactose fermented is converted into substances other than lactic acid. This is in agreement with the results of the previous

experiment with the difference that, while in that case the formation of by-products all took place in the early stages of the fermentation, in this case the by-products appeared to be formed steadily throughout the fermentation.

A parallel experiment was carried out with some of the same milk and starter as was used in the last experiment. To 900 ml. of the mixture was added a quantity of sterile calcium carbonate. Samples were taken periodically in a sterile manner and the mixture was shaken after each sampling. Acidity results, of course, could not be obtained. The results in this case indicated that the by-products were formed chiefly in the early stages of the fermentation, but as sampling errors were greater in this determination it is not possible to draw other than tentative conclusions.

Discussion.

With the starter organisms used, and where the fermentation of the lactose was allowed to proceed to completion, all but an insignificant amount of the lactose fermented appeared as lactic acid. At all stages of the fermentation the acidity developed in the milk corresponded closely to the amount of lactic acid produced. Where a starter containing more than one strain of organism was used, however, there was considerable formation of by-products in the early stages of the fermentation. Admittedly the experiment in question was not carried out under strictly sterile conditions, but in view of the large excess of starter organisms added it is not considered likely that the formation of by-products could be due to a contamination, more particularly since the side reactions did not continue through the experiment.

Summary.

1. Determinations of acidity, lactose and lactic acid were made at various stages during the souring of milk by starter cultures, both with and without the addition of a neutralising agent.

2. Throughout the fermentation, the increase in acidity of the milk was accounted for quantitatively by the amount of lactic acid produced.

3. In some instances in the early stages of the fermentation a considerable quantity of the lactose appeared to be converted to substances other than lactic acid.

4. Over the whole fermentation the amount of by-products produced from the lactose was small.

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BIENNIAL REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION A. PHYSIOLOGY OF DAIRY CATTLE

I. REPRODUCTION AND LACTATION.
II. NUTRITION.
III. GENETICS.

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I. REPRODUCTION AND LACTATION.

STUDIES in reproduction have for the most part been concerned with the hormonal activity of the reproductive organs or with pathogenic conditions of external origin affecting the pregnant uterus and its contents—the latter studies being outwith the scope of this review. There remains a review of the length of the gestation period of Holstein-Friesian cows by Knott(1) in which 2824 gestations are reported. The duration of gestation varied from 262 to 296 days, but 75 per cent. of all calves were born between the 275th and 285th days inclusive. Gestations of less than 270 days were observed in only 1·7 per cent. of cases, and of more than 290 days in 2·7 per cent.

Experimental evidence has been accumulating to show that quantitative undernutrition, vitamin A or E deficiencies, and mineral deficiencies may result in reproductive or lactational disasters. That the protein level of the diet must be maintained at a definite level to ensure the recurrence and normality of the oestrous cycle is suggested by Guilbert and Goss(2). Macomber(3) also stresses the importance of this factor in the maintenance of lactation. Breeding efficiency was not, however, diminished in a group of cows maintained over a period of 3 years on a diet containing much less calcium (0·18 per cent. dry matter basis) than is usually considered necessary(4), and Theiler(5) regards phosphorus deficiency as the greater menace to fertility.

Runge(6) reports researches on the ovaries of 128 non-pregnant and 96 pregnant cattle and describes the frequency of occurrence of ripe Graafian follicles, corpora lutea and cysts in each group. Numerous histological and anatomical studies are reported, among which may be noted those of Peitzer(7) and Schact(8) on the changes of the median uterine, the internal spermatic and caudal uterine arteries due to the functional requirements of pregnancy in cattle. The development of the mammary gland of the mouse, rat, guinea-pig and rabbit has been described(9, 10, 11), and Turner(12) has contributed valuable work on the anatomy and histology of the mammary gland of cattle including the foetal development. Tagand(13) describes the arterial, venous and lymphatic systems of the mature gland and stresses the extreme difference in diameter of the mammary artery as compared with that of the several mammary veins.

HORMONES.

The past two years have been notable for the widespread interest in the chemistry of the sex hormones. The conversion of cholesterol into the male sex hormone (androsterone) by Ruzicka *et al.*(14), the investigation of the oestrous-producing activity of a series of synthetic compounds related to oestrin by Cook *et al.*(15, 16), and the isolation of the pure corpus luteum hormone from extracts of this body(17, 18, 19) indicate the significant advances which have been made in this field. It seems possible that, by the use of standard preparations, co-ordination of work in the field of the hormonal

control of mammary development by the corpus luteum and placenta may yet be achieved.

The function of the anterior pituitary in the initiation of lactation is now recognised, and the presence of several separable hormones in the anterior hypophysis has been clearly demonstrated. While much confusion still arises in this field from the use of unstandardised extracts—as noted in the previous review—a certain amount of evidence based on hypophysectomy or the use of extracts of known reaction is available.

Many workers have been able to induce lactation by administration of hypophyseal extracts (20, 21, 22, 23, 24), and Riddle, Bates and Dyckshorn (20) have shown that their "prolactin" preparation is free from the other known effects of crude pituitary extracts, except that of inhibiting gonad development (25). The action of anterior hypophyseal extracts on the prolongation and yield of milk secretion has also been studied by Stocklausner and Daum (26) and by Azimov *et al.* (27). Further studies of the inhibition and stimulation of lactation by ovarian hormones have been carried out by de Jongh (28), but the precise co-ordination of hormonal activity is not yet clear. Turner and Frank (29) put forward the view that the joint action of oestrin and progestin is responsible for the development of the mammary glands and that hypophyseal "prolactin" then induces actual secretion. This view, however, has not been confirmed in all species. Full mammary development may be obtained in the male guinea-pig by the administration of oestrin alone (30) and hypophysectomy does not inhibit this action (31). Asdell and Salisbury (32) stress the necessity of progestin in mammary development, whereas Lyons and Catchpole (24) maintain that "prolactin" may also stimulate development. In general it seems that an ovarian factor is necessary in the first instance to prepare the gland, *i.e.* if interlobular tissue has once been formed under the influence of ovarian secretions, prepituitary extract will induce the formation of alveolar tissue even after ovariectomy.

That the pregnant uterus may stimulate although not maintain lactation in the absence of the hypophysis is suggested by the work of Collip *et al.* (33, 34) on hypophysectomised rodents, but suckling was not observed in the hypophysectomised cats of Allan and Wiles (35), although pregnancy and parturition were normal.

The part played by nervous stimulation in the secretion of milk has also received attention. Milk secretion has been observed in transplanted mammary tumours (36) —an observation which seems to discredit the role of nervous stimulation. Selye (37) and Selye, Collip and Thomson (38) offer convincing evidence, however, that the act of suckling may maintain "prolactin" secretion and hence milk secretion, but the path of the nervous reflex is unknown. The importance of the anterior hypophysis in endocrinology is such that further study of its innervation and of its general responses is urgently required.

Graham (39) has investigated the role of metabolic rate as a factor in the control of milk secretion and especially of the production of milk fat. Thyroidectomy and thyroid feeding were adopted to alter the metabolic rate of milking cows, and while moderate thyroid feeding of thyroidectomised and normal cows in late lactation temporarily increased milk yield, it had no effect in early lactation. The effect on fat production was parallel to but greater than that on milk yield. It is suggested that the action of thyroid on milk secretion is related to its effect on basal metabolic rate. In a later study (40) of the action of thyroxine on the milk and milk-fat production of normal cows, a rise in milk fat together with a somewhat variable rise in total milk secretion was observed. It is interesting to note, however, that neither di-nitrophenol nor "prolactin" had any appreciable effect on the mammary secretion. Further development of this work would be of value in the elucidation of the hormonal factors controlling milk secretion.

BIOCHEMICAL ASPECTS.

The study of the formation of milk in the active mammary gland must in the first instance depend on accurate qualitative data regarding the nature of the milk precursors. Rapid progress has been made in this field in recent years. In a detailed investigation of this subject, Lintzel(41), working on six goats and one cow, compared blood drawn directly from the left ventricle with that drawn from the mammary vein and in some respects also with that from the jugular vein. The criticism of the jugular-mammary comparison made by Blackwood and Stirling(42) has been upheld together with the role of blood sugar and blood amino acids as the precursors of milk lactose and milk protein. The phosphatide theory of milk-fat secretion has been shown to be unfounded by Lintzel and also by Blackwood(43), and evidence is advanced by the former which indicates that milk fat may be derived from the triglyceride fraction of the blood lipoids—a conclusion which seems to confirm the early experiments of Foá(44) on mammary perfusion. The phosphorus of milk—notably that of caseinogen—appears from the results of these two workers to be derived from the acid-soluble phosphorus compounds of the blood, principally from inorganic phosphate. The relative estimates found(43) of the requirements of the active mammary gland for glucose and inorganic phosphate agree closely. A high level of all blood lipoids seems to be required during lactation, while a gradual decrease is noted throughout its progress to a level which is markedly low during the non-lactating period. The results of Lintzel and of Blackwood, using slightly different methods, are seen to be in substantial agreement. Nitzescu(45), also investigating the nature of milk sugar precursors, was able to maintain the milk-sugar level of starving cows by intravenous injections of monosaccharides. Glucose was most efficient in this respect, whereas disaccharides such as saccharose or lactose were without effect.

A rise in blood sugar was produced experimentally by pumping glucose solution into the stomachs of lactating cows (Whitnah, Riddell and Hodgson(46)). The increase in blood sugar was marked in half-an-hour, and a return to the normal level was observed within 4–7 hours. Milk drawn at the time of maximum increase showed a higher lactose concentration than corresponding milkings on preceding or following days, but the subsequent decrease resulted in a subnormal lactose level. There was, however, no direct relation between increase in blood sugar and increase in milk sugar, so that blood-sugar level is not the primary factor in the regulation of milk-sugar production. The presence of organic phosphorus compounds in the mammary gland has been demonstrated by Borst(47)—the content being greatest in the virginal gland and successively lower in the gravid organism, in the active gland, and in the senile gland. An accumulation of organic phosphorus seems to occur between lactations. Adenosine phosphoric acid may be isolated from inactive glands, and a phosphatase, which can disrupt the phosphorus linkage, is also present in the gland. In surviving mammary tissue, however, inorganic phosphorus increases by autolysis more rapidly than lactacidogen is destroyed. Moreover, the production of inorganic phosphorus takes 6 hours for completion, whereas lactacidogen is destroyed in 2 hours(48). The significance of organic phosphorus compounds has also been investigated by Barrenscheen and Alders(49) in a study of the carbohydrate metabolism of the resting and active mammary glands of the rabbit and guinea-pig. The lactating gland has a higher phosphorus content than the resting gland. Organic acid-insoluble phosphorus compounds predominate—chiefly a hexose phosphoric ester which, since adenosine triphosphate is present, is presumed to be an intermediary in the synthesis of lactose by a process of preliminary phosphorylation. The inactive gland is shown to possess the power of breaking down lactose and galactose

to lactic acid. A synthesis of disaccharide has been observed by the action of preparations of mammary glandular tissue on glucose and galactose (Michlin and Lewitow(50)). This material has been isolated and is believed to be identical with milk sugar, but the stereochemical changes by which lactose may be formed *in vivo* from blood glucose are still unknown.

The principal constituents of milk are specific products of the mammary glands, and it is interesting to note that while lactose may be present in the urine immediately prior to or after parturition, Winter(51) could find no trace of lactose in the blood of lactating women. There can then be no threshold for lactose. Cutler and Lewis(52) found by the method of complement fixation that casein is present in the udders of all heifers after the 24th week of pregnancy, but in no udder of heifers pregnant less than 20 weeks.

Continuing their studies of the influence of dietary fat intake upon lactation, Williams and Maynard(53) have shown that the level of the various lipoids in the blood plasma of goats is affected by changes in the quantity of the fat fed, and that the nature of the fat fed on recovery from a fat-free diet determines the iodine value of the total blood lipoids. It would be interesting to know the relative rate of change of individual lipoids during lactation in such an experiment, and it is hoped that investigations of this nature will be pursued. When, however, the fat of a concentrate ration is replaced by an isodynamic amount of starch, no change is produced in the level of blood sugar either apparent or true (Turk and Work(54)).

Biophysical aspects.

In the last review of this subject attention was drawn to the controversy regarding the course of milk secretion between two milkings and to the striking results obtained by the consideration of the amount of milk obtained from amputated cow udders. From this work it appeared that milk secretion may be regarded as a continuous process, and that a large proportion of the milk obtained at any milking is present in the udder before the act of milking. This conclusion is confirmed and amplified by Swett, Miller and Graves(55). These workers compared the yield of milk from the amputated udders of eleven cows with the ante-mortem yields. The animals were divided into two groups, (1) in which the udders were allowed to become chilled after death, (2) in which the udders were maintained at approximately blood temperature until after the second post-mortem milking. The recovery of milk after amputation was 61·1 per cent. of the ante-mortem yield for group 1, and 75·3 per cent. for group 2. Approximately 80 per cent. of the total amount of milk recovered was obtained at the first post-mortem milking in both groups. In a second paper, Swett *et al.*(56) deal with the composition of the post-mortem milk. While temperature control played an important part in determining the amount of fat obtained from post-mortem milking, the highest post-mortem yield of fat was 40 per cent. of the total yield secured in the ante-mortem milking. Flushing out the udder with fat solvents failed to increase this value materially. It may then be said that post-mortem milk is normal except in respect of its very low fat content. The high fat content of "strippings" seems to indicate that the time and rate of fat secretion are not those of the total milk yield. Contributions to this subject have also been made by Isaachsen(57, 58), who points out certain fallacies attending the use of animals in advanced lactation. In a third paper Swett *et al.*(59) report that the capacity of the udder after amputation may be as great as 6 gallons, so that ample space is available for the storage of all the milk obtained at a milking. A study of the gross anatomy and histology of the glands employed is also reported. Petersen and Rigor(60) have determined the pressure developed by the accumulating secretion of milk during delayed

milking. Two measures of pressure were taken, (1) the maximum pressure developed in the udder by means of a manometer, and (2) the minimum air pressure required to arrest milk secretion. By the first method maximum pressure was developed 24–36 hours after milking, and thereafter cessation of secretion and resorption induced a marked fall in pressure. With the second method, secretion was inhibited by a pressure of 25 mm. of mercury. If milk is permitted to remain in the gland (61) until the maximum pressure is attained, marked alteration in composition takes place. The milk has a very low lactose and fat content together with a diminished calcium and phosphorus content, while protein is increased in amount but changed in character. The *p*H of the milk rises from 6·4 to over 7·3 in 120 hours. From a study of the effect of injections into the duct system of isotonic, hypertonic and hypotonic solutions and distilled water, these authors (62) conclude that the character and amount of secretion is regulated by the secreting cells and that there is no simple equilibrium existing across a semi-permeable membrane in the secreting cell. Secretion is best accounted for by a mechanism of selective absorption at the membranes separating the secreting cells from the blood stream on the one hand, and the milk ducts on the other.

The isotonicity of blood and milk has been the subject of frequent comment, and many studies have been made of the diverse levels of the substances which contribute to the osmotic pressure of each. The alveolar cells are apparently capable of adjusting to some extent the ash constituents of milk, but, in cases of serious disturbance, unaltered serum may be secreted in the milk. Nottbohm and Philippi (63) present an interesting study of the interrelations of sodium, potassium and chlorine in the blood and milk of cows and ewes. In two papers Folley and Peskett (64, 65) have studied the question of the permeability of the secreting cells of the mammary gland. They find evidence of correlation between the ratio of solids-not-fat to fat in milk, and the ratio sodium to diffusible calcium in blood. They consider that this factor may be responsible for the marked differences in milk composition which occur among the various breeds and between individual animals of the same breed. A study of the daily variations in composition of blood and milk failed to reveal any close relation between the two, but in general, high blood concentration parallels high milk concentration (Awdejewa (66)).

The four quarters of the udder vary in their yield of secretion—in general the fore-quarters give less than the rear-quarters, the latest ratio, according to Filipović (67), being 100:143.

Clinical chemistry of dairy cattle.

The study of normal values for various blood constituents has been reported by many workers. In particular, attention has been focused on metabolically active substances such as calcium, phosphorus, and sugar. Demmel (68), working with a large number of animals, reports the normal blood serum refractive index as 58·65 p.u., and notes the effect of oestrus, pregnancy and lactation. Frei and Demmel (69), in an extensive study of the influence of the sex rhythm on blood constituents, record a fall in calcium with advancing age, and as a result of castration, and a rise in calcium and glucose during oestrus. Teichmann and Logischen (70) report the effect of various factors on the blood sugar of thirty-one cows, and conclude that the blood-sugar level increases with advancing age, decreases with the progress of gestation and rises in the early stages of lactation to fall again as the milk yield increases. The diurnal variation noted by them for calves is very great. No correlation was observed between the blood-sugar level and any of the physical characters studied. Allcroft (71) records an interesting diurnal rhythm in the blood sugar of lactating cows, and em-

phasises the importance of time of sampling in long-range studies. In two papers Allcroft *et al.*(72, 73) record the effect of muscular exercise and/or excitement, adrenalectomy, and insulin treatment on the lactic acid, sugar and inorganic phosphorus of the blood of ruminants. Folley and Peskett(74) discuss the experimental factors associated with blood sampling which may cause variations in blood composition.

The importance of biochemical studies in the etiology of certain pathological conditions is now widely recognised, and the applications of such work should add materially to our knowledge of preventive medicine in relation to animal health. A comprehensive report of changes in blood composition of the cow during pregnancy and parturition has been given by Seekles, Sjollema and Kaay(75). Also, in relation to the increasing incidence of lactation tetany, Allcroft and Godden(76) have made a study of the normal changes in serum magnesium and calcium together with inorganic phosphorus in the blood of cows over parturition and of the young calf. Stare and Elvehjem(77) report the findings of phosphorus partition studies in the blood of normal and rachitic calves, and show the effect of vitamin D therapy in restoring a normal blood picture even on otherwise rickets-producing rations.

Regarding milk fever, two papers by Little and Mattick(78, 79) and one by Wilson and Hart(80) emphasise the association of low blood calcium levels with this condition. The former find that intravenous injection of calcium chloride solution is the only specific remedy of several investigated. Bell and Morris(81) find that the oxytocic power of cow's blood, while in general low, increases during the last week of pregnancy and falls again on parturition. Its power may be temporarily increased by the intramuscular injection of pituitrin. These workers(82) suggest a relation between the occurrence of milk fever in cattle and an extensive or continued secretion of pituitary hormone following parturition.

Factors affecting milk yield and composition.

The study of milk of animals other than the cow has been extended to include that of the yak by Amschler(83), the vixen by Young and Grant(84), the mare by Linton(85), and the buffalo by Shutt(86) and Tureanu(87). The fat components of the milk of Indian goats and sheep are reported by Dhingra(88), and Bomskov(89, 90) has determined the phosphorus partition in the milk of cows (stall-fed and grazing), goats and mares. Similar data for the human subject are given by Hess and Benjamin(91). The human milk studies of Macy and co-workers(92, 93) include the composition of 4-hourly samples of breast milk.

The composition of normal cow's milk throughout the year has been extensively studied and is reported by Golding, Mackintosh and Mattick(94) and by Baker and Cranfield(95). Seasonal variations in the composition of milk are also reported by Frambics and Szabados(96) and by Hickson and Thomas(97). Bartlett(98, 99) in two papers discusses the normal lactation curve and the variations in solids-not-fat with advancing age and pregnancy. In general, milk yield reaches a maximum at the sixth lactation, whereas the concentration of fat and non-fatty solids declines gradually from the first lactation. The occurrence of oestrus may produce fluctuations in composition, notably in fat content and certain physical characteristics(100). There is a close inverse correlation between the environmental temperature and the percentage of butter-fat in cow's milk, and this factor may be responsible for considerable variation in fat percentage between morning and evening milk(101, 102). The influence of climatic conditions generally is reported in detail by Houston and Hale(103). That factors operating at night have a tendency toward high milk production of low fat content is to be concluded from the work of Campbell(104).

The length of the calving interval may affect the average milk yield. While a

small gain may be expected in the current lactation from a short interval, the gain is offset by loss in the following lactation(105).

Recently an attempt has been made to treat the problem of milk low in solids-not-fat from the standpoint of criteria of normality and transudation phenomena. Lesser(106) reports the composition of such milks, while Peskett and Folley(107) consider that a dilution of the normal secretion of the udder by a fluid of the nature of oedema fluid may explain their production. Davies(108) regards milk of low solids-not-fat as composed of a true milk fraction and an "isotonic diluent". On the assumption of a 76 per cent. level of casein nitrogen in "standard" milk, the amount of the diluent and its nitrogen distribution and composition may be computed. When this is done the result compares with that of a modified blood or lymph serum or a casein and lactose-free lacteal secretion. The correlation of the history or presence of mastitis and the production of such milks is suggested.

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II. NUTRITION.

Physiology of digestion.

(*a*) *Saliva.*

During the past two years fresh evidence has been obtained regarding salivary secretion in ruminants. By means of parotid fistulae Trautmann and Albrecht(1) found that the parotid secretion was continuous and varied in adult goats up to 18·5 ml. in 5 min. In suckling kids only one-tenth of this volume was obtained. Whereas thermal and chemical stimuli had no effect, and psychic stimuli were of little account, mechanical stimuli increased the parotid flow, especially mastication. The larger the food mass or the more prolonged the mastication the greater was the secretion. Continued loss of saliva impaired the health of the animal and finally caused death unless 0·75 per cent. sodium bicarbonate solution was given. Examination of the secretion failed to reveal the presence of enzymes. Similar results have been obtained by Bechade(2). He found that mixed cattle saliva had no hydrolytic effect on raw or cooked starch.

Watson(3), continuing his earlier work on the nature and amount of phosphorus secreted in the mixed saliva, collected saliva by means of a sponge. The phosphorus, about 20–60 mg. per 100 ml., was all inorganic. Knowing approximately the amount of saliva secreted, Watson has estimated that 2·4–7·7 g. phosphorus are secreted daily.

(*b*) *Passage of food.*

The use of the rumen fistula has given more accurate information regarding the passage of food through the fore-stomachs of ruminants than was previously obtainable. Krywanek and Lampe (4), by means of thermo-electric couples inserted into the different stomachs of a 5-year old sheep through a rumen fistula, mapped the path taken by cold fluids by means of the fall in temperature. Fluid nearly always passed into the reticulo-rumen; only once, after copious water drinking, did the fluids pass directly into the omasum and abomasum. In young animals, on the other hand, Akssenowa (5) and Trautmann (6) found that the milk passed directly to the abomasum, being regurgitated back into the rumen. Akssenowa assumes that the abomasal enzymes remain active in the rumen. According to Trautmann the closing of the oesophageal groove is due to a reflex excited by stimuli coming from the pharynx, this reflex being uncertain with cold fluids. In a later publication Trautmann (7) found that regurgitation into the rumen from the abomasum occurred only in young suckling animals. More recently Ross (8) noted that a 1–2 per cent. copper sulphate solution passed directly into the abomasum in a higher percentage of cases than water.

By means of X-ray photographs Magee (9) examined the movements of food in the adult animal, after feeding pills composed of a mixture of $BaSO_4$ and $CaSO_4$ of varying specific gravity. To study food passage a drench was given after a fast of 15 hours. Large boluses formed in the mouth and were swallowed. Extraordinarily rapid movements then took place in the rumen, the bolus darting through the contents and becoming intimately mixed in a very short time. Heavy pills remained in the reticulum, while lighter ones passed into the reticulum and omasum. No regurgitation took place from the omasum, the pills being ground there to a very fine powder. Magee finally concludes that rate of passage depends on the specific gravity of the food.

(*c*) *Stomach movements.*

Magee (9) also examined the movements of all four stomachs. In the rumen and reticulum contractions took place in two stages, a sharp dorsal movement, a pause and finally a large contraction in a dorso-posterior direction. At the final contraction the outline of the reticulum disappeared, the contents being tipped into the rumen. A relaxation followed this movement. The period between contractions varied with the length of time after eating. Thus 4 min. after eating 47 sec. elapsed between reticular contractions, while 345 min. after eating the time was 77 sec. Differences due to age were also noted, a goat of 4 months showing an interval of 90 sec., whereas one of 9 months showed an interval of 40 sec. Churning took place by constriction of the muscular pillars, while contractions of the sacs were accomplished by peristaltic waves. The time between relaxation of one sac and contraction of the other varied from 2 to 30 sec.

Trautmann and Schmitt (10) studied the movements in suckling non-ruminating kids by X-rays and abdominal windows. They found that the rumen and reticulum were in continual movement. The contractions of the posterior blind sac were the most powerful, whereas the dorsal and ventral sacs alternated in contraction. The reticular contraction was biphasic, as in the ruminating animal. They concluded that reticular contraction was thus inessential for rumination.

Magee (9) found no omasal or abomasal movements, thus disagreeing with the earlier work of Schalk and Amadon (11). Movements in the fundus region of the abomasum were small, and it would appear that they were present merely to maintain a constant pressure on the food so that the pyloric region was always full.

Recently Popow *et al.*(12) have worked on the nervous control of omasal and abomasal movements. The vagus nerve was brought to the surface of the neck and the skin stretched to form a protective external cable. The nerve could thus be cooled or stimulated at will. Mild electrical stimulation of the vagus produced omasal contractions, while prolonged stimulation gave tetanic contractions. Subcutaneous injections of morphine did not eliminate the response, whereas pilocarpine caused an immediate arrest of all movements. The effect of stimulation in the abomasum depended largely on the functional state of the true stomach. By means of ice-water both nerves were excluded and the authors found that spontaneous movements immediately ceased.

(*d*) *Rumination.*

The movements taking place during regurgitation and rumination have for long been a controversial subject. Magee(9), by means of X-ray photographs, has considerably strengthened the earlier theory of Bergmann and Wester, that the respiratory tract plays an important part in the act. Magee found that the reticulum contracted as the diaphragm descended. The breath was held and a clear space appeared in the region of the oesophageal opening, apparently almost synchronous with the contraction of the dorsal ruminal sac. The clear space then disappeared and a mass of food shot up into the mouth. Chewing took place, at a rate depending on the rate of reticular contraction, since a reticular contraction always preceded rejection of the food mass. Contraction of the forepart of the rumen was, according to Magee, of more importance than that of the reticulum. This view had already been expressed in the earlier work of Czepa and Stigler(13).

Recently Jung *et al.*(14) connected the trachea to a bell-jar under reduced pressure, thus invoking a strong inspiration. Neither regurgitation nor any other sign of the rumination complex ensued. The authors concluded that reinforcement of thoracic inspiration is not alone sufficient to initiate rumination.

(*e*) *Effect of excision.*

In a detailed examination of rumen, reticular and omasal growth, Trautmann and Schmitt(15, 16) have obtained further evidence as to the mode of growth of these organs. The rumen and reticulum were excised from nine kids at an age of 2–4 weeks. X-ray examination and autopsy after 4, 6 and 8 months showed that regeneration had taken place, the size ultimately approaching that of normal goats. When roughage was fed soon after the operation regeneration was more efficient, whereas it was delayed if milk was fed for a prolonged period. This is somewhat contradictory to Trautmann's earlier work(17), which showed that kids fed to 7 months on milk died in a cachectic condition although the rumen, reticulum and omasum were perfectly normal. In this publication Trautmann(17) stated that no roughage was essential for rumen development. When rumen and reticulum were excised no rumination took place till a sufficient size was reached.

Trautmann(16) also found that after removal of the omasal leaves regeneration of a structurally sufficient wall took place when the animal was on solid food. The new leaves were less numerous, less thickly packed, shorter and thinner. They had, however, strengthened muscular layers and horny tubercles on the edges. On a milk diet regeneration was again less efficient.

(*f*) *Absorption.*

The possibility of absorption from the fore-stomachs has long been considered by physiologists, but no definite proof has been forthcoming. Recently Trautmann(18) re-examined the whole subject by a new method. He ligatured each stomach so that

no food could pass from one to the other. He then placed water and pilocarpine in one stomach and measured the salivary flow. By this means he found a marked and vigorous absorption in the three fore-stomachs, as marked as in the intestines. Abomasal absorption was very much slower.

(*g*) *Rumen Infusoria.*

Studying the temperature and *p*H requirements of the ciliates in the rumen, Koffmann(19) found that the optimum values were 39° C. and *p*H 5·5–8·0 respectively. *In vitro* the Protozoa die at 38–40° C. In certain sick cows where medicinal measures were unsuccessful, transference of Protozoa from healthy cows to the sick beast resulted in a rapid improvement. Koffmann concludes that there exists a relationship between the health of the animal and the protozoal content of the rumen. This suggestion is of interest in view of Trautmann's finding(17) that animals fed on a milk diet die. It appears that Trautmann's results may have been due to lack of rumen Protozoa.

Willing(20) attempted to cultivate rumen Infusoria by various means. Mechanical stimulation by shaking did not prolong the life of the Infusoria. Additions of casein and lucerne meal were favourable for growth of Infusoria, while starch and glycerine were unfavourable. Buffering, neutralising or reducing the acidity of the rumen content had little effect on the infusorial count. The ability of the Infusoria to live apart from the host has also received attention(21). Strelkow *et al.* found that when kept *in vitro* the organisms only survived for 1–1½ hours. These authors concluded that the only method of transferring Infusoria from one animal to another was from mouth to mouth or via common food or water.

The ability of the ciliates to digest food has been studied by Weineck(22), who found that starch and cellulose were digested, glucose being formed as the end-product.

(*h*) *Rumen bacteria.*

Woodman and Stewart(23) have obtained further evidence regarding the nature of the bacterial catabolism of cellulose. A culture of bacteria was obtained from sheep and was incubated with pure fibre and with various foodstuffs. With pure fibre the fermentation was found to be greater than with unextracted foods. This was attributed to the absence of ligno-cellulose from the pure fibre.

More recently Pochon(24) isolated cellulose-splitting bacteria from the rumen and studied the conditions for their optimal growth. The optimum *p*H was found to be 9·5 and the optimum oxidation-reduction potential was between 3 and 5.

Energy metabolism.

Considerable advances have been made during the past two years in our knowledge of the energy metabolism of ruminants, as regards both growth, pregnancy and lactation. It is only possible to give a very brief summary of the more important publications.

Brody *et al.*(25) undertook a detailed investigation into the relationship between resting energy metabolism and body weight of growing ruminants, from birth to 3 years. The metabolism was measured before the morning feed when the animals were resting normally, the metabolic rate being computed from the rate of oxygen consumption. The authors found the surface area law of little value in comparing the metabolism of different species, and relate growth metabolism to body weight by means of the exponential equations $Q/m = Ae^{-km}$ or $Q/m = Ae^{-km} + C$, where Q is the heat production for a body weight m, and C is the lowest or limiting value for Q/m.

Pregnant animals had a higher rate of metabolism than non-pregnant animals, while lactation gave a still higher value. Male cattle showed a 10 per cent. higher level than females, whereas after castration the two metabolic rates coincided. The difference between the true basal metabolism, after a fast of 48–96 hours, and the resting metabolism, after a 12-hour fast, was about 25–30 per cent., the higher value during the resting metabolism being due to the heat increment of feeding.

Mitchell and Hamilton(26) in a study of the utilisation of food energy found that this was partly controlled by the amount of food consumed. At the lowest levels of feeding all the nutrients were completely digested. The authors fed the animals at six levels, from fast to full feed. At the two highest levels the R.Q. > 1, while for the whole five levels of feeding the R.Q. values ranged from 0·88 at the lowest to 1·13 at the highest. The heat increment per kg. dry matter consumed increased from the lowest to the highest levels, whereas the net energy values decreased progressively. Mitchell and Hamilton conclude that there is a linear relationship between net energy and dry matter consumption. In a study of the food energy utilisation in lactating cows Forbes and Voris(27) fed nine Holstein cows for a complete year (covering both lactation and gestation) on as much food as they would eat. The most efficient animal converted 23 per cent. of the food energy to milk energy, while the average was 21 per cent. conversion for 313 days. A further study of the same problem by workers at the Pennsylvania Station(28) led to the same conclusions as Mitchell and Hamilton(26). A suggestive series of calculations from the same experiment, but published in a different journal(29), shows the proportion of energy liberated from the protein, fat and carbohydrate of the food at different nutritional levels. The proportion given by the protein would appear to remain constant, while the fat varies from 85 per cent. at starvation to 0 at maintenance, the carbohydrate being complementary to the fat. From maintenance to three times maintenance the proportion of energy from the carbohydrate remained constant and cost of fat synthesis negligible.

Recently Kleiber(30), working with two steers, published a long discussion on food energy utilisation. Briefly, the author concludes that, from the surface law, the economic use of food is not proportional to the size of the animal. Two animals are equal utilisers of food, if, with the same coefficient of production, their relative feed capacities are the same. The authors define coefficient of production as net energy divided by the difference between total food energy and food energy for maintenance, and relative feed capacities as the quotient of the maximum food energy which an animal can take in daily and its daily heat loss during starvation. In view of the present tendency to apply statistical methods to biological problems, it is evident that Kleiber has drawn conclusions from rather meagre data.

A mathematical discussion of the whole problem of energy utilisation has recently been published by Brody and Procter(31). They found that the net energy value of a feeding stuff varied with the gross energy intake according to the formula

$$Qn = A\,(1 - e^{-kQg}),$$

where Qn is the net energy value, Qg the gross energy intake, A the maximum net energy intake, and k the relative decline in the successive increment of Qn with increasing equal increments of Qg. The decline, they state, is due principally to increasing specific dynamic action with increasing plane of nutrition. An excellent review of the whole subject of specific dynamic action is also given. Another article by Brody(32) has appeared recently in *Ann. Rev. Biochem.*, in which he gives a summary of work to date on energy problems as regards both ruminants and non-ruminants.

PROTEIN METABOLISM.

(*a*) *The amino acids in nutrition.*

It has long been realised that the importance of any protein depends, for the major part, on the presence in its molecular structure of all the essential amino acids, *i.e.* those which the animal body cannot synthesise. Since the greatest part of the research work into the subject of the essential amino acids has been undertaken with small animals, it seems necessary to outline briefly the more important advances in all branches, whether the experimental work has been carried out with ruminants or not.

The essential nature of arginine and histidine has been a controversial subject since the first publications of Hopkins in 1916. Recently Rose and his co-workers (33, 34) have re-studied the question. They investigated the increase in tissue arginine during growth and compared it with the arginine intake of rats upon an arginine-low diet. The increase in tissue arginine was two to three times the amount fed, a definite proof that it is non-essential. In a later experiment they showed that addition of arginine gave no increase in the growth of rats. The five carbon amino acids, ornithine, glutamic acid, hydroxyglutamic acid, proline and hydroxyproline, were found to be non-interchangeable. The essential nature of phenylalanine has also been proved (35), but no definite conclusions regarding the indispensability of tyrosine have yet been reached (35, 36). The essential nature of lysine and tryptophane has long been recognised.

With ruminants the subject has necessarily to be attacked in an indirect manner (38, 39). For a lactating cow it is clearly impracticable to feed pure proteins deficient in one or other amino acid. The method used was therefore to determine in the more important food proteins those constituent amino acids which have been shown by work on small animals to be essential for growth. The technique employed in these analyses (37) was slightly modified from the original method of Grindley *et al.* (41). Feeding experiments were then undertaken, using in one series foodstuffs low and high in lysine content, as compared with milk, and in the second series foods with varying quantities of tryptophane (38, 39, 40). It was concluded, from the fall in milk yield and the increased urinary excretion of nitrogen during those periods in which a ration deficient in either lysine or tryptophane was fed, that both these amino acids are essential for milk production in the ruminant.

(*b*) *Non-protein nitrogen.*

The utilisation of non-protein nitrogen has again received the attention of the German workers during the past two years. Although the evidence advanced is very inconclusive the publications warrant attention.

Ehrenberg and his co-workers (42, 43) have attempted to show that ammonium salts can replace proteins for both maintenance and milk production, the ammonia being converted to bacterial and infusorial protein in the rumen. In the first experiment only one-half of the nitrogen required for maintenance was fed as protein, the remainder being made up with ammonium salts. The milk yield and nitrogen content of the milk remained normal over the period, about 10–14 days. No loss in weight was noted. It has, however, been shown (38, 39) that there is a lag period when the ration of a cow is changed from an efficient to a deficient protein, the milk yield sometimes remaining normal up to 16 days, although the urinary nitrogen may show marked changes. Unfortunately Ehrenberg did not analyse the urine.

The second series of experiments was very much larger. In this two groups of eight cows were fed on the same basal ration of silage, hay and wheat. To one group

was added a production ration of soya beans, decorticated earth-nut cake and sunflower cake, while the other group received rice meal, molasses and ammonium bicarbonate. In both groups the production ration has a total S.E. of 7·68 kg. and a P.E. of 1·47 kg. for a milk yield of about 20–22 litres; this corresponds to about 4 lb. S.E. and 0·80 lb. P.E. per gallon. The authors conclude that since there were no marked differences in the fall in milk yield (group I fell from 22·4 to 11–19 l., and group II from 20·5 to 10–16 l.), the ammonia must have been converted to protein. Unfortunately the feeding standards used for both maintenance and milk production were far in excess of actual requirements, and there would thus be protein in the maintenance ration capable of use for milk production. Moreover, if the beet silage were made from young beet tops, it is probable that the amino-acid constitution of the beet protein would be of value for milk production.

Kirsch and Jantzon(44, 45) have also attempted to show that both amides and ammonium salts could partially cover the nitrogen requirements of lactating cows. The ration fed consisted of clover silage, molassed beet slices, potato flakes and ammonium bicarbonate. No fall was noted in milk yield, but, as in the experiments of Ehrenberg, the amounts of protein fed appear to have been excessive.

(*c*) *Nitrogen metabolism.*

Since Carpenter's publication on the fasting steer in 1927 no work of importance has been published on either the fasting or the endogenous metabolism of the ruminant. Several publications on the nitrogen metabolism of growing and lactating animals have been published. Ashworth and Brody(46) analysed the urine from ten cows between the ages of 7 and 40 months. No indication is given of the nitrogen intake or the type of protein fed, but the statement is made that the animals were fed according to "good dairy practice". The urea plus ammonia nitrogen constituted 79 per cent. of the total nitrogen, of which the ammonia nitrogen was almost negligible, the preformed creatinine nitrogen 3·6 per cent., and the total creatinine nitrogen 6·5 per cent. The creatinine coefficient was 9·5, remaining constant during the entire age interval, and the creatine coefficient was 7·6, this also remaining constant. The ratio of calories (basal metabolism) to mg. preformed creatinine nitrogen declined steadily with increase in live weight from 3·3 at 6 months to 1·4 at 40 months. One important fact is evident from these results, *i.e.* that the energy requirement for maintenance decreases with increase in live weight, whereas the protein requirement remains practically constant, if the ratio of creatinine nitrogen to endogenous nitrogen is constant.

Detailed urinary analyses have been undertaken with lactating cows by Morris and Wright(38, 39). The proteins fed during the course of the investigations varied in their "quality", some being deficient in either lysine or tryptophane, whereas others were, as far as is known, complete. The amount of protein fed in the production ration was only slightly in excess of the actual requirements for milk production (0·44 lb. P.E. per 10 lb. milk). In those periods in which complete proteins were fed the urea nitrogen constituted about 60 per cent. of the total nitrogen, ammonia nitrogen 15 per cent., preformed creatinine nitrogen 3·6 per cent., and creatine nitrogen 2 per cent. When foods deficient in one essential amino acid were fed the urea nitrogen constituted about 50 per cent., ammonia nitrogen 30 per cent., preformed creatinine nitrogen 2·8 per cent., and creatine nitrogen 2 per cent. The total nitrogen output in the urine varied with the different rations fed, being on the deficient proteins almost double that on those periods in which the complete proteins were ingested, although the total nitrogen intake in all cases was the same. The nitrogen balance results showed little, except that, on the whole, positive balances were recorded throughout,

due probably to the state of rest in the metabolism stalls following a period of activity on the farm. The high output of ammonia nitrogen was explained by the authors as being due to an excess deamination leading to the formation of large amounts of organic acids which would require neutralisation.

Dann (47) undertook an examination of the urines of grazing cows when different supplements were given, *i.e.* meadow hay and brewers' grains. No reliable conclusions can be drawn from the results, since collection was made from only one urination on any one day, no figure being given for the total daily urinary output. Differences were noted between the urinary nitrogen values on the two supplements, but it is impossible to assess the significance of these.

Das Gupta (48) has noted that, irrespective of the benzoic acid content of a ration, the whole appeared in the urine as hippuric acid. This would explain the abnormally high hippuric acid nitrogen results frequently found when certain rations are fed.

A study of the nitrogen partition during late pregnancy and over the puerperium has been made, using goats as the experimental animals (49). The partition in the goat was found to differ from that in the cow, the percentage of urea nitrogen being about 80 as compared to 60 in the cow. It is of interest to note, however, that shortly before parturition an increase in total nitrogen output was found, together with an increased percentage of ammonia and creatine. Post-partum there was a quick return to normal excretion. A later paper (50) on the effect of injections of posterior hypophysis extracts on goats showed that the effect was very similar to that occurring at parturition. The author concluded that a hypersecretion from the posterior lobe of the hypophysis probably took place prior to parturition, causing both the abnormal metabolism and expulsion of the young.

Mineral metabolism.

Much work has been done during the past few years on the mineral requirements of ruminants. Only brief mention of the various publications can be given. Ellenberger (51), using ten cows, found positive Ca and P balances throughout a complete year. The negative balance found at the commencement of lactation rapidly became positive. Hart *et al.* (52) fed two groups of twenty-two cows throughout growth and three lactations on rations containing varying amounts of Ca and P, one group receiving 28 g. Ca and 28 g. P daily, the other 158 g. Ca and 74 g. P. Seven cows from each group were killed and the bones analysed. The mineral reserves in both groups were the same, showing the minimal supplemental requirements necessary for ruminants. Turner *et al.* (53) obtained similar results, *i.e.* that supplements are unnecessary if the rations fed are of high quality. The effect of varying the Ca : P ratio on the absorption of Ca and P has been studied by Otto (54). When the ration was low in P larger amounts of Ca were eliminated by the kidneys, while additions of Na_2HPO_4 aided the absorption and retention of Ca. When $CaCO_3$ was added to a diet low in P there was a reduced absorption of P. The utilisation of proteins, fats and carbohydrates was unaffected. The effect of Na_2HPO_4 has also been noted by other workers (55, 56), while Riddell *et al.* (57) have fully confirmed the effects on Ca excretion of a diet low in P. Becker *et al.* (58) noted a marked increase in milk secretion when bone meal was added to a diet low in Ca, showing that minerals can act as limiting factors for milk production. Lintzel (59) emphasises the importance of Ca and P content, the Ca : P ratio, and the K and Na content of the diet. He states that an excess of Ca and Mg over P_2O_5 is necessary, good meadow hay being taken as a standard. An attempt to produce an acalcicosis by diets low in Ca was found by Du Toit *et al.* (60) to be unsuccessful, whereas an aphosphorosis was readily produced on diets with a low P content. They also concluded that with normal medium pasture grass no K, Cl or Mg deficiency could be produced.

The effect of iodine on the prevention of abortion has been studied(61, 62). Forbes *et al.*(61) took twenty-five cows infected with, or which had been exposed to infection with, *Br. abortus*. The cows were given 1·2 g. of iodine per head per day over a period varying from 8 to 73 days. No effect on the incidence of abortion was noted. Thomson(62) found that addition of iodine to a diet low in this element maintained the health of the animals together with their milk yield. Scharrer *et al.*(63) also observed the marked effects of feeding an iodine-rich grass as compared with a grass containing only small quantities of iodine. A higher milk yield, a butterfat of higher iodine value, a more prolonged lactation period, and favourable effects on the oestrous cycle were all noted.

The whole question of the mineral requirements of cattle is closely related to mineral deficiency diseases, which are comparatively common in semi-arid regions, such as South Africa and certain of the American States. The subject may be briefly dealt with under three headings:

(*a*) *Phosphorus deficiencies* (*osteomalacia and rickets*). Wellmann and Marek(64) found that true rickets and osteomalacia could be induced in ruminants by feeding rations with unsatisfactory mineral components. Vitamin D in any form would prevent the disease. In animals a simultaneous fibrous transformation of the bone marrow together with osteoclastic dissolution of the bones were marked symptoms. These authors claimed that a marked excess of Ca over P produced rickets, whereas Becha(65) stated that osteomalacia was a disturbance of the Ca metabolism and laid particular stress on the effects of the Mg content of the ration on Ca excretion. Theiler(66) found that both rickets and osteomalacia were due to an aphosphorosis, vitamins having no effect. Later(67) he examined microscopically the bones from heifers on a P-deficient ration and noted a marked atrophy of the bony tissue—osteoporosis, accompanied by a marked increase of osteoid tissue corresponding to the degree of P-deficiency. An admirable review of the whole subject has been given by Theiler(68).

(*b*) *Calcium deficiencies.* Very few workers have succeeded in causing a deficiency disease in cattle by feeding a Ca-deficient ration. Reed and Huffmann(69) fed a low Ca diet (0·278 per cent. Ca) for 5 years and found that normal growth and reproduction were obtained, a result which was confirmed by Fitch *et al.*(70). Theiler(67) and Du Toit *et al.*(60) stated that the production of an acalcicosis is almost impossible in cattle.

Orr *et al.*(71), continuing the earlier work of Elliot and Crichton(72) on the calcium-deficiency theory of "Bentleg", fed sheep on a Ca-poor pasture, and found that emaciation and bone deformity occurred. Cod-liver oil caused immediate disappearance of the symptoms. Auchinachie and Fraser(73) suggested that this is more closely associated with a faulty Ca : P ratio than with a calcium deficiency *per se*. No similar bone abnormalities have been induced in cattle.

(*c*) *Iodine deficiencies.* Poor development of cattle and sterility are very common in iodine-deficient areas(74, 75). Danger, however, lies in overdosage with this element(76, 77), a high mortality in the young and destructive changes in the ovaries being noted.

The exact mode of action of iodine is unknown. Kelley(78) found that iodine caused an increase in the absorption and retention of N, P and Ca. Tunnicliff(79) noted that a marked increase of phagocytosis in the blood occurred after intravenous injection of sodium iodide. Probably both mechanisms assist in restoring health and fertility.

FEEDING EXPERIMENTS.

(*a*) *The effect of ingested fat on butterfat production.*

Allen(80) noted an immediate rise in butterfat production when whole milk or cream was added to a ration. Later he found(81) that almost any fat could increase the milk fat, the increase being proportional to the amount fed and occurring within 24 hours of feeding. Unfortunately the experimental periods are only 6 days, and it has long been recognised that animals can, with change of ration, give a transient increase in butterfat production. Honcamp *et al.*(82) note similar results. Sutton *et al.*(83), using corn oil, could not confirm Allen's results nor could Sheehy(84). The latter claimed that the increase in butterfat production when whole milk was fed was due to some substance other than the fat in the ration.

Maynard and McCay(85, 86), using nineteen cows fed benzene-extracted foods, found that when the fat ingested was only 3 per cent. of the ration a fall in milk yield only was noted, while at 1 per cent. the butterfat also fell. They advise a fat intake equivalent to the fat secreted in the milk.

The effect of various types of fats has also been examined. Petersen(87) confirmed the fall in butterfat yield after feeding cod-liver oil. A rise in butterfat yield after feeding ethereal oils (oleum anisi) has been noted by Ringeisen(88), while Bünger *et al.*(89) could obtain no effect on either milk yield or butterfat percentage by the addition of crude lecithin to the ration.

(*b*) *The effects of various rations on milk yield.*

The effect of irradiated dried yeast on milk yield has been widely investigated, but, irrespective of the food which it replaced, no change in milk yield was noted (90, 91, 92, 93, 94, 95). Comparisons of rations containing fresh and dried sugar beet pulp indicate that neither the yield nor the quality of milk is affected by replacing the fresh by the dried material(96, 97, 98, 99, 100, 101). The effect of palm kernel cake on milk yield has been investigated; an increase in yield and butterfat percentage has invariably been observed(102, 103, 104). Recently considerable attention has been given to the nutritive value of dried grass and the new acid-treated (A.I.V.) silage. As regards dried grass Camburn(105) and Newlander(106) found that, when this product partly replaced hay and grains, no change in milk yield was noted. When dried grass replaced hay alone a rise in milk yield was found whereas when it replaced the full concentrate ration a fall occurred. As regards A.I.V. silage, Virtanen(107) reports that silage made from various crops was found to be of high nutritive value. It is understood that a number of experiments are in progress in this country to determine the value of the method in conserving grass. It will be interesting to see how these results compare with those obtained in Finland and Sweden.

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III. GENETICS.

General.

Since the last biennial review some 200 papers have been published dealing principally, or in large part, with the inheritance of milk yield. This does not include references to other than genetic causes which affect the lactation yield, and which must be taken into consideration in a genetic study. Such factors are age, age at first calving, service period, dry period, etc. In order to overcome variations in yield due to changing environment, which must occur even in one and the same herd, Von Patow reported what is known as the "byre average" which, roughly speaking, compensates for many of these factors. Buchholz(1) tested this theory on data obtained from a group of small herds, with a view to demonstrating whether or not the calculation of a common byre average for a group of herds is practically possible. His results supported Von Patow's conclusions that milk yield is transmitted by three independent homomeric genes. Apart from correction for seasonal variation, the yields were corrected only for age. Schubert(2) also investigated the reliability of the byre average. He finds that this expresses satisfactorily the variations in yield due to weather and feeding. Hündsdorfer(3) applied the byre average to a primitive breed. He reaches the conclusion that high yield in improved breeds may be due to a series of mutations, and claims that 20 per cent. of the total variability in yield falls to the byre average, and 60 per cent. to genetic causes, while all other known genetic causes amount to 20 per cent. Working with the yields of 170 cows, he showed that Von Patow's theory of three independent homomeric genes holds good.

Axelsson(4) follows the group of investigators who maintain that the number of genes involved is so great that the effect of each of them can neither be fixed nor followed from generation to generation. He believes that it is unlikely that an exact gene analysis leading to simplification in breeding can be accomplished. In a later paper(5) he studies the correlation coefficient between daughters and dams which, on a corrected system, he calculates for total milk yield to be $r = 0{\cdot}211$, and for butterfat percentage $r = 0{\cdot}386$. Plum(6) working with registered Jersey cows in a single herd, found that the influence of the sire appears to be only a little greater than all other factors which change from year to year. Heizer(7), analysing the butterfat production of dams and daughters, obtained the remarkably high correlation of $r = +0{\cdot}778$. He was working with a herd of pure-bred Ayrshire cattle. He decides that within the herd the sire's influence is negligible and varies in ability to transmit milk productivity factors to his progeny. Krizenecky(8) correlated data on the subject of milk yield and absolute butterfat production in relation to fat percentage. He finds that the variation of milk yield and of absolute fat yield is practically the same, the average coefficient of variability being 1:1·037. The variation of fat percentage is much lower, being only 40 per cent. that of milk yield. There is always a high positive correlation between milk yield and absolute fat yield. He finds this correlation to be $r = +0{\cdot}8432$. He obtains a small negative correlation which is barely significant between milk yield and fat percentage. The same author(9), by treating the data in another manner, confirms the foregoing and reaches the conclusion that milk yield has a decisive influence upon fat yield. Other aspects of butterfat production are discussed by Solovjev(10) and Sutton and Washburn(11), who deal with a comparison of the milk fat values of butterfat from four breeds of dairy cattle on similar feed.

The existence of sex-linked factors is discussed by Taufer(12). Working with two breeds, the Steppe and the Red East Friesian, he comes to the conclusion that the

inheritance of fat but not milk yield is in part the product of sex-linked factors. He quotes the work of Bogdanor in support of these conclusions.

Fohrman and Graves (13) in an analysis of the advance registry records of fifty-one Ayrshire cows found no evidence of a sex-linked character. Support for this theory might be obtained from the work of Copeland (14), who analysed the productivity of the granddaughters of bulls according as to whether their grandsire appeared on the paternal or maternal side of the pedigree. Copeland, however, thinks that the difference may be due to influences in the selection of the parental stock.

Persistency of yield is a character of considerable importance. Davydov (15), working with the Jaroslav breed, obtained a coefficient of correlation of $r=0{\cdot}75$ of maximum daily yield and lactation yield, but a much lower correlation of persistency with total yield. He found that the average degree of persistency varied in different herds. He also describes the effect of environmental factors, etc., upon persistency. Gaines (16) also deals with this subject.

The fat globule has been the subject of an extensive piece of work by Campbell (17). He has produced a competent review of the whole subject and has shown that many factors, of which heredity is but one, cause variation in the size of the fat globule. Amongst these are injury, illness, stage of lactation, and food. His data are taken from the Bowlker herd at Illinois, comprising Holsteins and Guernseys, together with their F_1's and F_2's. This material was also supplemented from the Wisconsin herd of Angus and Angus-Holsteins. The mean fat-globule volume of individual Guernsey cows is found to be more variable than that of the Holsteins. The Angus appear to show a higher mean fat-globule size than does the Guernsey. The F_2's resultant from the Guernsey × Holstein cross and from the Angus × Holstein cross showed a more rapid rate of decline in the fat globule during the first 6 weeks of lactation than did the pure-bred Guernseys or Holsteins. A significant difference was found between the means of the Guernseys and the Holsteins and between the Guernseys and the F_2's of this cross. The conclusion is reached that a number of factors appear to be involved in the inheritance of this characteristic, and that these multiple factors act like those determining milk production rather than those determining the percentage fat content.

The vitamin content of milk has been the subject of investigation by many authors, some of whom have considered the genetic aspect, which, however, does not appear to be of much importance. Gunderson and Steenbock (18) come to the conclusion that the vitamin B content of milk is not subject to breed variation. Wilbur, Hilten and Hauge (19) dealt with the vitamin A activity of butter produced by Guernsey and Ayrshire cows. The activity of the samples was found to be similar, regardless of the breed of the cows and the colour of the butter. They conclude that the higher the percentage of fat, the greater the vitamin A activity of the milk. Hathaway and Davis (20) conclude that, under the conditions of their experiment, Holstein cream contained more vitamin A than Jersey cream.

Owing largely to the work of Hill (21, 22), attention is being focused on the hardness of the curd of milk. The technique employed is interesting and has been the subject of criticism by other workers in this field. Nevertheless, the results of these studies do indicate that there exists a major breed difference in respect to the hardness of the curd. There also appears to be some evidence that related cows, such as dams and daughters and paternal half-sisters, have a fairly high correlation as regards curd tension tests.

Conformation.

Conformation in relation to milk production is always a subject that has fascination for those who study the inheritance of milk yield, if only because there is a

possibility that it may lead to some means of phenotypic selection of young stock. Gowen(23) has continued his studies on this subject, and deals with American Jersey Registry of Merit cows. He has correlated yield and butterfat yield with size and various other measurements. He finds that the age of the cow is always related to her milk production and to her conformation in such a manner that, as age advances, the milk production and the size increase up to mature form. This joint increase of milk yield and size with age creates a partially spurious correlation between milk yield and conformation. This can be best eliminated by judging only within age classes. Likewise the weights of dairy cattle are related to their milk secretion in such a way that a relatively large weight means a relatively large flow of milk. The food intake necessary to maintain this larger size may, however, partly or wholly overcome the advantage of weight. Gowen considers that this depends, in part, on the measure utilised in determining the economy of production. At the same time he maintains that cows should show relatively little fleshing at the withers. He lays emphasis upon the milk veins and the udder. In a further paper(24), the same author deals with the conformation of the parents as related to the milk secretion of their daughters in the same breed. He finds that, with the possible exception of weight, there is no correlation of practical significance between the body type of the sire or the dam and the productive capacity of the daughter in milk, butterfat or butterfat percentage.

Garner(25) has made a like study based on Shorthorn cows in England and Friesian cows in Minnesota. He obtained slight positive correlations between high milk yield and body length, body height, and circumference at chest and barrel. Between the size of milk wells and milk yield he obtained a correlation of $r = 0{\cdot}37 \pm 0{\cdot}027$. He draws the conclusion that the total area of the milk wells of a cow is an excellent indicator of her milk-producing ability, and states that, with a little experience, farmers may quickly find and measure milk wells with their fingers. Usually a large milk well will take the tip of the middle finger of a normal man's hand. Although one should never attempt to judge a cow by one point alone, the size of the milk wells is probably the best single point indicative of milk-producing ability. There may be a close relationship between the size of the milk wells in bulls and the milking capacity of their female progeny.

Chiodi(26) attempted to ascertain the total surface of the epithelium of the mammary glands in cattle in different herds characterised by a different capacity for milk production. Forty-three mammary glands were removed from heifers at 60–75 days of age. The results suggest that total glandular development varies directly with the capacity for milk production.

Swett, Miller, Graves and Creech(27) study the quality, size, capacity, gross anatomy, and histology of udders. They find that the producing utility of cows appears to be moderately correlated with size, and distinctly correlated to the capacity of udder, but were unable to show any relationship to other udder characteristics. There was a positive correlation between udder quality and the post-mortem recovery of milk. Prouty and Ellington(28) have also dealt with the physical characteristics of the cow's mammary system in relation to production. Lüthy(29), working with the Swiss Simmental breed, has calculated various indices of conformation. Davydov(30) has studied the extent of white markings as influencing yield in the Holmogor breed, and finds that, after grouping 577 cows according to the extent of white markings, no correlation existed between markings and yield. An excellent description of the show points of cattle and their relation to productivity has been published by Nevens and Kuhlman(31).

PEDIGREE, BREED CONSTRUCTION, INBREEDING, ETC.

The construction of the Ayrshire breed is dealt with by Fowler(32). He obtained no difference in the coefficient of inbreeding as between registered bulls and cows. He also found that high milking cows showed a remarkably lower coefficient of inbreeding than the breed average. On the other hand, when the coefficient of inbreeding of high-yielding cows (over 1000 gallons) was compared with that of cows giving average yields (under 800 gallons), it appeared that inbreeding itself had no detrimental effect on milk yield. The paper includes some observations on Wright's "Coefficient of Inbreeding", and emphasises the profound influence which may be exerted in a breed by one herd, or indeed one bull.

Tansky(33) finds that there has been little line or inbreeding practised by the Holstein-Friesian breeders of the United States. However, the records of the outstanding sires of that breed show a modicum of line-breeding.

Woodward and Graves(34) report an experiment begun in 1912 by the U.S. Department of Agriculture. Two bulls were used on a herd of grade cows. Their progeny were inbred to them. Inbreeding continued in these two lines but was discontinued in one owing to lethal factors and poor yield. The other line still exists, and the yields of the fourth and fifth generations are better than those of the outbred daughters of the original bull. This experiment shows, amongst other points, that inbreeding *per se* is not antagonistic to high milk production. Plum(6), dealing with a single Jersey herd, finds that within groups of daughters by the same sire, it would seem that the more inbred daughters tend to be the lower producers. He finds that slightly more than one-fourth of the variation of production is determined by crosses which vary from year to year, or with different sires used.

Stautner(35) has made a study of the blood lines and the transmission of certain desirable characters in the Bavarian spotted cattle. Wilsdorf(36) puts forward an ingenious suggestion for the registration of animals in a tabular form, male and female lines being inbred along the two axes, and the progeny in the squares of intersection. He contends that the data provided is thus clear, concise, and complete.

The subject of the influence of the pure-bred and the grading up of stock is one which continues to receive attention. Lush(37) has published an interesting description of the improvement of grade dairy cattle affected by this means in Louisiana. Kuhlmann, McGilliard and Weaver(38) describe similar work in Oklahoma. Likewise there is a report from Wyoming(39). LaMaster(40) deals with the cattle of South Carolina. Wilkinson and Walker(41) have done similar service in respect of records in Australia.

Copeland(42, 14) has studied pedigree records with a view to determining those records which are of the greatest assistance in the selection of bull calves. He comes to the conclusion that the records of the sire's daughters are of considerably more value than is the record of the dam alone. On the evidence submitted, the records of the daughters of the maternal grandsire are more closely related to the production of the grandson's daughters than are the records of the daughters of the paternal grandsire. He states that it would appear that, if a cow has a production record herself, and if she has two or more tested daughters, and if in turn her sire has a number of tested daughters, the sum of this information gives a good index of her germinal composition. He stresses the importance of getting all possible information regarding the milk yields of the families in the immediate ancestry of a bull-calf. His two papers include some interesting relationship correlations which are in accord with previous work of this nature. Hewitt(43) provides similar data from Australia.

Heizer(44) describes an inherited udder abnormality. White and Ibsen(45) report the occurrence of an inherited form of acute mastitis and incline to the belief that other forms of mastitis may also be due to a genetic diathesis.

Awdejewa and others(46) have made a study of the correlation between the composition of blood and of milk, using Schwyz and Yaroslav cattle. They did not obtain any direct parallelism in the variation of the composition of blood and of milk. High blood concentration corresponds to high milk concentration, but the possibility of the influence of blood composition upon the milk secretion is not excluded. The two breeds studied differed in respect of chemical composition and the freezing-point of blood and milk. Diem(47) has made a study of the dry matter of blood and its relation to constitution and performance in the spotted mountain cattle.

The progeny test.

It is symptomatic of the attention which has been focused recently upon the proven sire that approximately half of all the references relating to the inheritance of milking capacity deal with this subject. Strictly speaking, these papers dealing with the progeny test do little or nothing to advance our knowledge of the mode of the inheritance of milk yield. Nevertheless, the progeny test is a potent means for the improvement of dairy cows; the manner of its application is involving much discussion and the application of scientific method. The need for some form of progeny test may be gauged from a report by Wintermayer(48), who studied the yields of the daughters of 1280 American bulls scattered throughout 296 Bull Associations in twenty-six different States. He reports that one-third of these bulls leave their daughters giving appreciably less milk than the dams of those daughters. He finds that only one-third of these registered bulls showed an increase of over 50 lb. in the daughters' yields as compared to their dams'. Many other papers have been written to show the practical value of the progeny test, and it is not proposed to refer to these further, except so far as they may contain other material on the subject.

At the present moment dispute concerning the application of the progeny test appears to be centred round two points. The first is the number of daughters required to provide data of sufficient accuracy. The second is the best manner (if any) by which a "bull index" may be computed. As most of the papers deal with both these aspects, and as they are interrelated, it is not proposed to separate the one from the other in the following discussion.

Warwick and Copeland(49) propound a plan whereby the value of a bull should be assessed by mating him to low-yielding cows. To be tested adequately, a bull would have to be mated to fifty low-producing cows, and test herds should be kept. In theory there is something to be said for this procedure. Yapp(50) maintains his attitude in favour of a sire index based on the yields of the daughters plus the difference between them and their dams, thus giving credit to a bull capable of increasing yield. Lush(51) brings considerable additional information to bear on the problem, and focuses attention on certain aspects which there has been a tendency to neglect. Dealing with the suggestion of an index based on the average of the yields of the daughters alone, he states that if each herd could be regarded as a random sample of the cows in the breed, the average merit of the herds would not be exactly the same, yet the differences would not be important. This leads him to decide in favour of the index put forward by Norton which is similar to that of Yapp(50), except that it takes into account the average amount of regression toward the mean of the breed which has occurred in the past without bothering to inquire into the causes of that regression. Lush points out that the greatest vulnerability is in the possibility that the daughters and dams may not have been tested under the same conditions, and that

a minor weakness is the failure of this system to utilise information about daughters out of untested dams. Lush does not discard the daughter average as useless as an index. He states that it is the first step on which usable sire indexes are all built, but it neglects differences in the merits of the dams and is rather sharply affected by herd environment. With regard to the number of daughters required, Lush states that the records of more than three daughters give more information than can possibly be obtained from the most complete study of the pedigree of a dairy bull. It is undesirable to publish any sire index based on fewer than five daughters, and more than this number are required if the dams are not taken into account.

Wright(52) brings to the problem a long experience in the practical aspects of the breeding of animals, combined with a sound knowledge of biometry. He points out that there are several quite different questions which one may ask, all of which relate to the same sire. The first question relates to the record to be expected from a given mating. This is a practical question, but the answer is not an evaluation of the sire. The second deals with the expectation of the yields of future daughters based on the knowledge of the sire's past performance, and assuming that he continues to be used in the same herd. This is an evaluation of the sire plus dams at a certain level of production. The third question deals with the record to be expected of daughters of a bull if the latter were mated to a random sample of cows of the breed. The answer to this question is the only one that could, according to Wright, be used as the basis for an evaluation of the sire, and herein lies the explanation of many of the discrepancies and arguments on this subject. Since, in practice, different bulls have different opportunities, any attempt to obtain an unbiased evaluation demands that all of the sires to be compared be mated to comparable groups of cows. To meet these points, Wright produces a formula which, however, cannot be truly applied in practice.

Graves(53) emphasises the importance of knowing all the available records. He believes that the dam's production must also be taken into account. Fohrman and Graves(13), in their study on the progeny of fifty-one Ayrshire sires, state that, in order to evaluate the transmitting ability of a proven sire, it is necessary that a complete analysis be made of the performance of all his daughters, giving due consideration to environmental influences. They doubt the value of correction factors to equalise such influences. They believe that the selection of a sire depends on an intelligent study and careful analysis of performance records from every aspect. They are also of opinion that "no so-called bull index in existence to-day is so formulated as to afford a guide to constructive breeding procedure. At best, the bull index gives only a relative assay of the value of an animal as a sire".

In Great Britain Edwards(54) has investigated this problem. Dealing with the minimum number of daughters required to prove a sire, he calculates the variance of one daughter from the formula (for all groups) of the sum of squares of deviations from the group mean divided by the total number of daughters less the number of groups. This is done on the records of ten daughters to each of seven bulls. His results are published in a table, which shows the decline in the standard deviation as the number of the progeny increase. Thus, with three daughters, the standard deviation is 1063 lb. and 13·7 per cent. of an average yield. With six daughters these figures are reduced to 751 lb. and 9·7 per cent., whereas with fifteen daughters they are 475 lb. with 6·1 per cent. He thus comes to the conclusion that with fewer than about six daughters, the figure of significant difference is too large for the attempt to mean much. He admits that none of the numbers is really satisfactory, and grants that it is a further argument for the necessity of standardising the conditions under which records for proven bulls are being made. He then continues an investigation of the various bull indexes, and concludes that the average yield of the daughters is the most satisfactory indicator for practical purposes.

Mackintosh (55), to whom must be given the credit for being the first amongst the more modern Agricultural Advisers to advocate the progeny test as a means of improving the productive qualities of our dairy cattle, has produced an excellent report upon the progeny of four bulls in the herd of the National Institute for Research in Dairying. An interesting point is an illustration of a fact, realised by many breeders, that the rate of maturing of the progeny of one bull is different from that of another. Thus, the actual mature yields of the daughters of one bull were some 16 per cent. higher than the yields as calculated by the use of correction factors. He has been able to test the accuracy of six daughters as an index from a practical point of view. With the bull having the largest number of daughters, the first six gave actual yields amounting to 716 gallons. The second lot averaged 654 gallons and the third lot 548 gallons. The average of thirty-two daughters was 618 gallons. From a study of his data Mackintosh concludes that, so far as milk yields are concerned, the differences between the averages for the first groups of six daughters from each of the four bulls might be due to chance. There is no convincing evidence that any one bull was definitely superior or inferior to the others. Under such circumstances other qualities possessed by the progeny, namely fat percentage, shape and potential wearing qualities of the udder and teats, breed type, etc., would be the deciding factors if it was necessary to place this group of bulls in order of merit. From a consideration of the yields of the dams he concludes that information obtained from the dam/daughter comparisons does not add materially to the knowledge of the bull already gained from the average yields of the first group of unselected daughters.

Davydov (56), dealing with bulls of the Kholmogor breed, puts forward a proposition not unlike that of Warwick and Copeland (49). He gives what he considers to be standard rates of improvement for "good bulls", and advises the estimation of the yield by a study of the shape of the lactation curve of the daughters. Ivanova (57) has calculated that 140 animals is the optimal number required to test a sire. This means that in a dairy breed 140 heifers are required, and that the bull should be mated to 350 cows. Serebrovsky and Ignatyev (58) have devised a method of plotting the most probable "quality" of the best animal. From this they deduce that, for any character studied, the higher the correlation the less will be the influence of environment. They assume three cases, and plot trebled quadratic errors of the values for daughters along three corresponding curves. On this basis it is possible to discuss the best number of bulls to test on a given number of cows. If 200,000 cows are available, and if for a character for which the bulls are being tested $r = 0{\cdot}25$, then when 3500 bulls are used, the trebled quadratic error of the deviation for the best group of daughters from mean "quality" will be greater than the deviation itself.

Larsen (59) reports on the progeny testing of Danish bulls. He refrained from using correction factors or bull indices, and concludes that the first test, based on eight to nine daughters, does not materially differ from a later test with fifty or more daughters, a marked difference only being obtained in eight out of fifty-eight cases. Larsen demonstrates the necessity of a progeny test, but states that speculation upon the right "bull index" is not the crucial problem. What is important is to have as many farmers as possible recording the heifers of bulls still in use, and acting accordingly. Axelsson (5) has published the results of progeny testing in the Swedish-Friesian breed.

Peters (60) discusses the Mount Hope index and points out that it is undesirable to use only old proven sires, and that in selection both conformation and pedigree should be taken into account. Zorn and Krallinger (61) also discuss the Mount Hope index, and recommend Wilsdorf's hereditary grid system in which the performance of the dam is shown on one axis and that of the daughter on another. Voitellier (62)

discusses the various types of bull index from those of Gowen down to the present day, and their applicability to problems in France.

Smith(63) has reviewed the problems concerning investigations into the inheritance of milk yield both from practical and economic aspects. He considers that selection alone will not effect the desired improvement in productivity such as has been shown to be capable of achievement with crops such as maize. Further fundamental knowledge is essential, otherwise the rate of improvement is bound to get slower. He states that breeders should not be led to expect too much from the progeny test; otherwise there is danger of a popular reaction. Lush(64), discussing this paper, makes some interesting observations upon the effect of selection, especially in relation to the number of replacements in the breeding herd. He points out that only genetic variance, which can be expressed additively, is subject to simple mass selection. "For instance, if one-third of the variance in the milk yield of a number of dairy cows is genetic in this narrow sense of the word, and if the breeders pay no attention to bulls but select the cows so intensively that those saved for breeding average 150 gallons more than their entire contemporary generation, one can expect the next generation to average about 25 gallons above the generation in which their parents were born. When compared with the 150 gallon *selection differential*, the 25 gallon gain may appear disappointingly small. No doubt this explains the fact that in every country there are sometimes raised the voices of zealous enthusiasts proclaiming that the old methods of selection and attention to pedigree are useless, but that the latest panacea of progeny test, sire index, or what not, will accomplish all which the old methods are condemned for not doing."

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Since writing the above, another useful paper has come from Dr J. L. Lush(65) dealing with the progeny test. In the first place he points out that it is incorrect to say that a progeny test is genotypic and therefore dependable, whereas a cow's own record is phenotypic and therefore not dependable. No fundamental definition is really involved, since the progeny test itself consists of some kind of an average of phenotypic records of the progeny. "To suppose that the process of averaging would purge these phenotypic records of all their error, and would bring out only the pure gold of genotypic truth, seems to require too much faith in the potency of arithmetic!"

The paper really seeks to answer the question as to whether a cow's own record, or the average record of n of her daughters, is the more dependable indicator of the cow's own breeding value. The solution, under the simplest conditions, is presented algebraically and graphically. Under these conditions there must be at least five offspring before the progeny test in a whole population will usually be a more accurate indicator of the parent's breeding value than is the parent's own performance. If the parents, or the records used to represent them, are more highly selected than the offspring or their records, then the progeny test may become relatively more accurate than under the simple conditions for which the algebraic solution is given.

Lush concludes that the progeny test is needed most for traits which cannot be expressed in one sex, and for traits which are but slightly hereditary. He adds that there are three foundations on which to estimate the breeding value of an animal. These are: pedigree, own performance, and the progeny test. As fast as some selection is practised on one of these methods, the possibility for further progress by additional selection on the same method rapidly diminishes; correspondingly, increased attention should be given to one of the other methods.

Attention is drawn to the danger involved in the suggestion that bulls could be properly tested by mating them to low-yielding cows. The assembling and maintaining of a herd of recessive cows for such tests would be expensive. Nor would this method avoid deception by epistatic effects. The author concludes that it is unlikely that such a plan would yield information worth the cost of getting it. If the sire and dam belonged to different breeds, there would be increased probability of important misleading epistatic effects, which would probably preclude using for testers individuals of a breed developed for other purposes, as for example the use of beef bred cows for testing the breeding value of a dairy bull for milk and fat production.

Dealing with the progeny test for bulls, Lush writes: "For traits which the sire cannot express himself, there is nothing but a pedigree estimate of the sire against which to compare the accuracy of a progeny test of him. Since a progeny test surpasses even the best pedigree estimate when there are more than three progeny (provided the offspring do not resemble each other very much for any other reason except that they are by the same sire), the progeny test in such cases is much more useful and more urgently needed for the sire than for the dam. However such progeny tests will rarely tell as much about the sire as the available information correctly used will tell about a dam of nearly equal age. Enthusiasm over some such slogan as the current one that 'The next best thing to a proved sire is the son of a proved sire' should not cause us to forget that such a son is one generation of Mendelian segregation away from this sire and that half of his inheritance (a little more than half if the probably small amount of sex-linked inheritance in mammals is also considered) comes from his dam whose breeding value may usually be estimated more closely than that of his proved sire, if the available information is fully and fairly used."

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